Over-expression of pumpkin GA-oxidases in *Arabidopsis thaliana*

Vom Fachbereich für Biowissenschaften und Psychologie
der Technichen Universität Carolo-Wilhelmina
zu Braunschweig

Zur Erlangung des Grades einer
Doktorin der Naturwissenschaften
(Dr. rer. nat.)

genehmigte
DISSERTATION

von
Abeer Ahmad Farag Mahmoud Radi
(M. Sc. In der Pflanzenphysiologie)
aus Ägypten

1. Referent: Prof. Dr. Theo Lange

2. Referent: Prof. Dr. Ralf-R. Mendel

Eingereicht am:

Mündliche Prüfung (Disputation) am:

2005

(Year of Printing)

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung des Fachbereichs für Biowissenschaften und Psychologie, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Publikation:

Radi A, Lange T, Niki T, Koshioka M, Pimenta Lange MJ. Ectopic expression of pumpkin gibberellin oxidases alters gibberellin biosynthesis and development of transgenic *Arabidopsis* plants. Submitted for publication in The Plant Journal.

Tagungsbeiträge:

Frisse A, Schmidtke S, Radi A, Pimenta MJ, Lange T. Gibberellins and the regulation of plant development. (Presentation). 2nd International Conference "Molecular Analysis of Phytohormone Action", Hamburg, (March 2002).

Radi A, Kappler J, Fischer A, Pimenta MJ, Lange T. Gibberellins and the regulation of plant development. (Presentation). 4th International Conference "Molecular Analysis of Phytohormone Action ", Freising, (April 2004).

Radi A, Pimenta MJ, Niki T, Koshioka M, Lange T. Over-expression of pumpkin GA-oxidases in *Arabidopsis thaliana*. (Poster). In: Book of Abstracts. Botanikertagung, Braunschweig, Abstr. No. P01-23 (September 2004).

Fischer A, Kappler J, Radi A, Padeffke T, Pimenta MJ, Lange T. Gibberellins and the regulation of plant development. (Presentation). 5th International Conference "Molecular Analysis of Phytohormone Action ", Würzburg, (April 2005).

The work described here was carried out between October 2000 and January 2005, in the Department of Plant Physiology and Biochemistry (Institut für Pflanzenbiologie der TU Braunschweig, Germany) in partial fulfilment of the requirements for the Degree of Ph.D.

I gratefully acknowledge my indebtedness to Prof. Dr. Theo Lange, Head of the Department of Plant Physiology and Biochemistry, who greatly influenced my scientific progress and dedicated most of his time and energy helping his research students. I am glad express my sincere appreciation and gratitude to him for his keen supervision, suggesting the problem and following my progress throughout the course of this investigation.

For kindly agreeing to act as a co-referee, I am grateful to Prof. Dr. Ralf-R Mendel.

Gratitude is also due to Dr. Maria J. Pimenta for her participation in supervising this work, most helpful suggestions, invaluable advises, and permanent motivating support were of inestimable value.

I also want to express my thanks to all co-workers of the institute for their readiness to help, the fruitful discussions which contributed to the success of the work and for the wonderful working atmosphere which was positively reflected on the smooth running of the work.

I wish to extend my gratitude to the Egyptian government for financial support.

I wish to express my gratitude and sincere thanks to my family, husband Mokhtar and sons Omar and Ali for giving me love, continuous moral support and inspiration, I needed to accomplish successfully this work.

Table of Contents

List of Abbreviations

aq:	Aqueous
AS:	Antisense
bp:	Base pairs
C:	Carbon atom
cDNA:	Complementary-DNA
CIAP:	Calf intestinal alkaline phosphatase
CPP:	*Ent*-copalyl pyrophosphate
CPS:	*Ent*-copalyl pyrophosphate synthase
CTAB:	Cetyltrimethyl ammonium bromide
dATP (A):	2´-Deoxy-Adenosine-5´ triphosphate
dCTP (C):	2´-Deoxy-Cytidine-5´ triphosphate
dGTP (G):	2´-Deoxy-Guanidine-5´ triphosphate
dTTP (T):	2´-Deoxy-Thymidine-5´ triphosphate
dNTPs:	2´-Deoxy-Nucleotide-5´ triphosphates
DEPC:	Diethyl pyrocarbonate
DMSO:	Dimethyl sulfoxide
DNA:	Deoxyribonucleic acid
DNase:	Deoxyribonuclease
DTT:	Dithiothreitol
E.coli:	*Escherichia coli*
E-cup:	Eppendorf-cup
EDTA:	Ethylene diamine tetracetic acid
ER:	Endoplasmic reticulum
ETOAc:	Ethyl acetate
EtOH	Ethanol
fg:	Femto gram
g:	Gram
GA_n:	Gibberellin A_n
GAs:	Gibberellins
GA-ox:	Gibberellin-oxidase

GAP	Glyceraldehyde-3-phosphate
GC-MS:	Gas chromatography-mass spectrometry
Gent:	Gentamycin
GGPP:	*Ent*-geranyl geranyl pyrophosphate
GUS:	β-Glucuronidase
h:	Hour
H_2O_{bidist}:	Bidistilled water
HoAc:	Acetic acid
HPLC:	High performance liquid chromatography
IPP:	Isopentenyl pyrophosphate
KA:	*Ent*-kaurenoic acid
Kan:	Kanamycin
KAO:	*Ent*-kaurenoic acid oxidase
KO:	*Ent*-kaurene oxidase
Kb:	Kilobase pairs
KS:	*Ent*-kaurene synthase
LB:	Luria-Bertani-medium
M:	Molar (mol/liter)
min:	Minute
MCS:	Multiple cloning site
MeOH:	Methanol
mRNA:	Messenger ribonucleic acid
MS:	Murashige-Skoog-medium
NTPs:	Nucleotide-5´ triphosphate
OD_n:	Optical density, n=nm
PCR:	Polymerase chain reaction
pg:	Pico gram
Rif:	Rifampicin
RNA:	Ribonucleic acid
RNase:	Ribonuclease
RNasin:	Ribonuclease-inhibitor
rpm:	Rounds per minute

RT-PCR:	Reverse transcriptase-polymerase chain reaction
S:	Sense
SDS:	Sodium dodecyl sulphate
sec:	Second
TAE:	Tris-Acetate-EDTA
T-DNA	Transfer-DNA
Ti:	Tumor-inducing
TE:	Tris-EDTA
T_m:	Melting temperature
Tris:	2-Amino-2-(hydroxymethyl)-1,3-propandiol
U:	Unite
UV:	Ultraviolet
V:	Voltage
v/v:	Volume per volume
WL:	Wavelength
WT:	Wild type
w/v:	Weight per volume

1. Introduction

Plants produce signalling molecules, called phytohormones, which play a major role in plant development and regulate growth at low concentrations. Some hormones are produced in one tissue and transported to another tissue, where they initiate specific physiological responses. Other hormones act within the same tissues where they are produced. In both cases, they affect the developmental or physiological state of cells, tissues, and in some cases, separated organ systems.

The word hormone comes from the Greek *horma* meaning “to set in motion”. For a long time, it was thought that hormones act only by stimulating plant growth. However, recently, it has been found that some of them have inhibitory functions. Therefore, it is more useful to regard them as plant growth regulators. Nevertheless, this term also needs qualification because the response of a particular regulator depends not only on its chemical structure but also on how it is “read” by the target tissue.

There are five generally recognized classes of plant hormone. They are all organic compounds and include auxins, gibberellins, cytokinins, ethylene, and abscisic acid (Kende and Zeevaart, 1997). Recently, it has been suggested that brassinosteroids, salicylic acid, and jasmonates are major classes (Creelman and Mullet, 1997). The brassinosteroids, which are complex organic molecules related to steroids, were found to be required for normal growth of most plant tissues. Salicylic acid has been implicated as a signal in defence responses to plant pathogens. The jasmonates volatile compounds, recognized as components of floral fragrances, are now known to act as regulators of plant development.

1.1. Discovery of the gibberellins

The observations of White in 1917, led to the conclusion that there is a certain factor, which controls the length of pea plants (White, 1917). This factor was considered responsible for the regulation of plant growth, and was then later proposed to be GA. The beginning of research on gibberellins can be dedicated to Japanese scientists who investigated the causes of the “bakanae” foolish seedling disease, which had

destructive effects on the rice economy. In 1935, Yabuta succeeded in isolating a chemical compound from the fungus *Gibberella fujikuroi* that stimulated shoot elongation in rice. This compound was named gibberellin. Subsequently, two fungal growth active compounds, which they termed gibberellin A and gibberellin B were successfully isolated (Yabuta and Sumiki, 1938).

In 1954, a new gibberellin, named "gibberellic acid", was separated and had physical properties different from those reported by the Japanese gibberellin A (Curtis and Cross 1954). In addition, another three components were extracted from the methyl ester of gibberellin A. These components were nominated gibberellins A_1, A_2, and A_3. Gibberellin A_3 was confirmed to be identical to gibberellic acid (Takahashi et al., 1955).

Brian and Hemming (1955) found that the application of GA_3, obtained from culture of *G. fujikuroi*, promoted dwarf pea plants to a normal growth and concluded that dwarf pea do not contain GA_3. Radley (1956) demonstrated that this substance, purified from tall pea plants, induced stem elongation of dwarf plants and proposed that GAs are produced naturally in higher plants. Similar observation was reported in dwarf maize using plant-extracted substances with GA-like activity (Phinney et al., 1957). In the same year, a new gibberellin was isolated and named gibberellin A_4 (Takahashi et al., 1957). This was followed by the separation of crystalline gibberellin A_1, A_5, A_6 and A_8 from *Phaseolus multiflorous* (MacMillan and Suter 1958; MacMillan et al., 1959, 1960, 1962). After 1968, all gibberellins were assigned numbers as gibberellin $A_{1\text{-}x}$, regardless of their origin (MacMillan and Takahashi, 1968).

The *ent*-gibberellane skeleton is the base to all gibberellins structures (Figure 1).

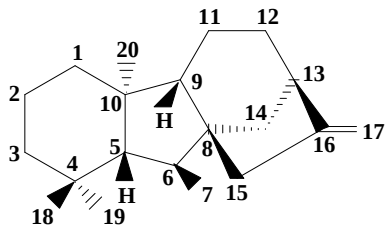


Figure 1: *ent*-Gibberellane structure

Gibberellins can be divided into two different groups: the C_{20}-gibberelins (C_{20}-GAs) that contain twenty carbon atoms and the C_{19}-gibberellins (C_{19}-GAs) that have one carbon atom less. There are other differences in the basic structure, especially the oxidation state of carbon 20 and the number and position of hydroxyl groups on the molecule.

Gibberellins are involved in all stages of plant development: they promote stem elongation, seed germination, leaf expansion, flowering, and fruit development (Crozier, 1983; Hedden and Proebsting, 1999; Richards et al., 2001; Olszewski et al., 2002). Furthermore, it has been shown that cell elongation and cell division were affected by the level of GAs (Kende and Zeevaart, 1997). The activity of the catalytic enzymes involved in the gibberellin biosynthetic pathway regulates the levels of the bioactive GAs, which are responsible for the control of growth and plant development. Therefore, genetic manipulation of these enzymes could serve as a tool for controlling plant growth, which has a wide application in agricultural fields (Hedden and Kamiya, 1997; Lange, 1998; Phillips, 2004).

Gibberellins are chemically identified as natural tetracyclic diterpenoid carboxylic acids made up of four isoprenoid units. The development of chemical and analytical techniques made it possible to isolate and identify a large number of gibberellins in plants, fungi, and bacteria. Although, more than 126 different gibberellins have been identified, only a small number of them are considered bioactive GAs, the others are supposed to be precursors or degradation products (Hedden and Phillips 2000a). Based on the facts described above, it is generally concluded that gibberellin biosynthetic pathway is highly complex (Graebe, 1987; MacMillan, 1997; Sponsel and Hedden, 2004).

In higher plants, GA_1, GA_3, GA_4, GA_5, GA_6, and GA_7 are considered to be bioactive gibberellins. They were found to promote plant development in certain bioassays and thus described as plant hormones (Graebe and Ropers, 1978; Hedden and Phillips, 2000a). The complete biosynthetic pathway of gibberellins was assessed in seed and vegetative tissues of several plant species using radioactive precursor determination (Kobayashi et al., 1996).

1.2. Gibberellin biosynthetic pathway

Gibberellin biosynthetic pathways can be differentiated into three major stages according to the localization and the nature of the enzymes involved. In the first stage of the pathway, *ent*-geranylgeranyl pyrophosphate (GGPP) is converted to *ent*-kaurene in the plastid. In the second stage, *ent*-kaurene is oxidized by cytochrome P450 mono-oxygenases at the endoplasmic reticulum (ER) to form GA_{12}-aldehyde. Finally, GA_{12}-aldehyde is converted to bioactive GAs by soluble 2-oxoglutarate-dependant dioxygenases in the cytosol (Figure 2; Graebe, 1987; Hedden and Kamiya, 1997; Lange, 1998; Hedden and Phillips, 2000a).

1.2.1. Stage 1: Production of terpenoid precursors and biosynthesis of ent-kaurene

Isopentenyl pyrophosphate (IPP), the basic biological isoprene unit that is used in gibberellin biosynthesis, is generally synthesized in plastids from glyceraldehyde-3-phosphate (GAP) and pyruvate (Lichtenthaler et al., 1997). However, in the endosperm of pumpkin seeds, IPP is formed in the cytosol from mevalonic acid (Graebe, 1987; MacMillian, 1997). Isopentenyl pyrophosphate is converted to *ent*-geranylgeranyl pyrophosphate (GGPP) by only two enzymes, IPP-isomerase and GGPP-synthase, followed by the conversion of GGPP to *ent*-copalyl pyrophosphate (CPP) and finally to *ent*-kaurene. Both steps require two diterpene cyclases, CPP synthase (CPS) and *ent*-kaurene synthase (KS). The cyclization step catalysed by CPS is promoted by protonation of C-14-C-15 double bond of GGPP. This is classified as a Type-B cyclization, while conversion of CPP to *ent*-kaurene is initiated by ionisation of the diphosphate (Type-A cyclization). The two enzymes that catalyse the reactions are found in the proplastids of meristematic shoot tissues, and they are not present in mature chloroplasts (Aach et al., 1997).

1.2.2. Stage 2: Oxidation reaction at the ER to form GA_{12} and GA_{53}

In the second stage of gibberellin biosynthesis, *ent*-kaurene is transported from the plastid to the endoplasmic reticulum, and is oxidized enroute to *ent*-kaurenoic acid (KA) by *ent*-kaurene oxidase (KO), which is associated with the plastid envelope (Helliwell et al., 1999; 2001b). A methyl group on *ent*-kaurene is oxidized to a carboxylic acid, followed by contraction of the B ring from a six to a five-carbon ring to give GA_{12}-aldehyde. All the enzymes involved are cytochrome P450 mono-

oxygenases and localized at the endoplasmic reticulum. GA_{12}-aldehyde at C-7 is then oxidized to GA_{12}, which is the first gibberellin in the pathway and thus the precursor of all other gibberellins. Some gibberellins in plants are also hydroxylated on carbon 13. The hydroxylation of C-13 of GA_{12} originates GA_{53}.

1.2.3. Stage 3: Steps after GA_{12}-aldehyde

The third stage of the GA biosynthetic pathway involves 2-oxoglutarate-dependent dioxygenases (7-oxidase, 20-oxidase, 3-oxidase, and 2-oxidase) which results in several side reactions and different GA products. This can be attributed to the fact that dioxygenases have multiple functions depending on the nature of their substrate (Lange and Graebe, 1993; Hedden and Kamiya, 1997). These enzymes are classified as non-haem iron-containing oxygenases and oxidases (Prescott, 1993; De Carolis and De Luca, 1994; Barlow et al., 1997).

In pumpkin, the C-7 of GA_{12}-aldehyde is oxidized by a soluble GA 7-oxidase (CmGA7ox), which was isolated from developing pumpkin seeds, resulting in GA_{12} in addition to the mono-oxygenase activity (Lange et al., 1994b; Lange, 1997), but there are some mono-oxygenase genes in other plant species. This enzyme further converts GA_{12} to GA_{14}, which is considered to promote an early 3β-hydroxylation pathway (Graebe, 1987; Frisse et al., 2003). In all systems known, GA_{12} is converted by GA 20-oxidase and GA 3-oxidase enzymes in two different branches; namely non-13-hydroxylated pathway and early 13-hydroxylated pathway, resulting in bioactive GA_4 and GA_1, respectively. In the 13-hydroxylated pathway, GA 13-oxidase converts GA_{12} to GA_{53} to form the 13-hydroxylated metabolites. GA_{12} and GA_{53} are further converted by GA 20-oxidase to GA_9 and GA_{20}, respectively. This occurs by the oxidation of C-20 to an aldehyde followed by the removal of this C atom and formation of a lactone. GA 20-oxidase1 from developing pumpkin seeds (CmGA20ox1) catalyses the oxidation of GA_{12} to GA_{15}, GA_{24}, and GA_{25}, and GA_{53} to GA_{44}, GA_{19}, and GA_{17}, respectively (Lange, 1994; 1998; Lange et al., 1994b; Frisse et al., 2003). In some species, GA_9 and GA_{20} are also converted to GA_7 and GA_3, respectively (Albone et al., 1990). The bioactive GAs, GA_4 and GA_1, are then formed from GA_9 and GA_{20}, respectively, probably as side reactions of GA 3-oxidase activity (Spray et al., 1996).

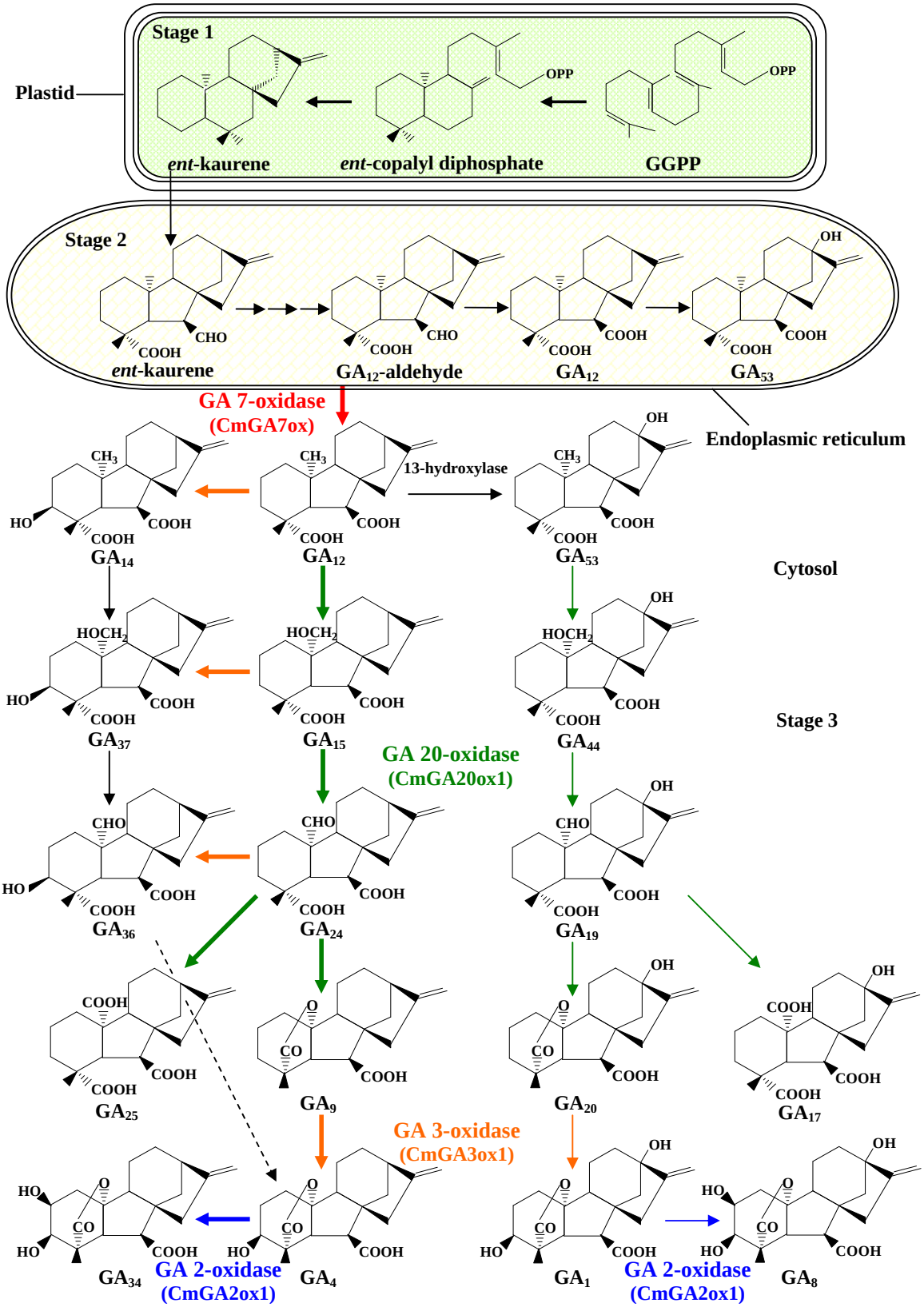


Figure 2: The three stages of gibberellin biosynthesis in higher plants. Bold arrows indicate major pathways in developing pumpkin seeds.

In pumpkin endosperm a bi-functional GA 3-oxidase1 (CmGA3ox1, previously called 2β,3β-hydroxylase, Lange et al., 1997b) catalyses both steps, 3-oxidation and 2-oxidation. Moreover, 3-oxidases from other plant species work mainly on C_{19}-GAs but CmGA3ox1 prefers C_{20}-GAs as the substrate (Lange et al., 1997b; Hedden, 1999). In addition, GA 3-oxidase1 converted GA_{12}, GA_{15}, and GA_{24} to GA_{14}, GA_{37}, and GA_{36}, respectively. GA 2-oxidase1 (CmGA2ox1) from pumpkin deactivates GAs by 2β hydroxylation to form GA_8 and GA_{34} (Figure 2, Frisse et al., 2003).

1.3. Gibberellin biosynthesis enzymes and their encoding genes

The discovery and establishment of the biosynthetic enzymes involved in gibberellin biosynthesis made it possible to investigate the genes encoding their biosynthesis. These encoding genes were first isolated from *Cucurbita maxima* and *Arabidopsis thaliana* (Table 1). Eventually, many homologous genes were cloned from other plant species, e.g. *P. sativum, S. oleracea, Z. mays* (Hedden and Kamiya, 1997; Lange, 1998; Kang et al., 1999; Yamaguchi and Kamiya, 2000; Hedden et al., 2002; Olszewski et al., 2002).

In *Arabidopsis*, the enzymes that are involved in the early stage of the pathway (CPS, KS, and KO) were found to be encoded by single copy genes (Sun et al., 1992; Sun and Kamiya, 1994; Yamaguchi et al., 1998; Helliwell et al., 1998). In the second stage, the P450 mono-oxygenase, *ent*-kaurene oxidase (KO) was encoded by the *GA3* gene of *Arabidopsis* (Helliwell et al., 1998). On the other hand, the dioxygenases controlling the final stages of the pathway are encoded by multigene families: five GA 20-oxidase (Phillips et al., 1995; Xu et al., 1995), four GA 3-oxidase (Sponsel and Hedden, 2004) and eight GA 2-oxidase (Thomas et al., 1999; Schomburg et al., 2003; Phillips, 2004). The functions of GA 3-oxidase genes (Williams et al., 1998; Yamaguchi et al., 1998), three of the GA 20-oxidase (Phillips et al., 1995) and five of the GA 2-oxidase genes (Thomas et al., 1999) have been confirmed through the expression of their cDNAs in *E. coli*. However, GA 2-ox5 is not been expressed and is proposed to be a pseudo gene (Table 1). In some cases, the concentration of bioactive GAs was altered when these genes were over-expressed in transgenic plants. This indicates that the regulation of these genes is important in controlling the late stage of the pathway (Hedden and Phillips, 2000b; Yamaguchi and Kamiya, 2000).

Table 1: Genes involved in GA biosynthesis, including their function and reported from Hedden et al., 2002 and Lange, 1998.

Plant species	Gene	Enzyme function	Reference
A. thaliana	*CPS*	CPP synthase (GGPP to CPP)	Sun and Kamiya, 1994
	KS	*ent*-kaurene synthase (CPP to *ent*-kaurenoic acid)	Yamaguchi et al., 1998
	KO	*ent*-kaurene oxidase (*ent*-kaurene to *ent*-kaurenoic acid)	Helliwell et al., 1998
	KAO1	*ent*-kaurenoic acid oxidase (*ent*-kaurenoic acid to GA_{12})	Helliwell et al., 2001a
	KAO2	*ent*-kaurenoic acid oxidase	Helliwell et al., 2001a
	GA20ox1	GA 20-oxidase ($GA_{12}/_{53}$ to $GA_{9}/_{20}$)	Phillips et al., 1995; Xu et al., 1995
	GA20ox2	GA 20-oxidase	Phillips et al., 1995
	GA20ox3	GA 20-oxidase	Phillips et al., 1995
	GA20ox4	Undetermined	
	GA20ox5	Undetermined	
	GA3ox1	GA 3β-hydroxylase ($GA_{9}/_{20}$ to $GA_{4}/_{1}$)	Chiang et al., 1995; Williams et al., 1998
	GA3ox2	GA 3β-hydroxylase	Yamaguchi et al., 1998
	GA3ox3	GA 3β-hydroxylase	Phillips and Hedden,
	GA3ox4	GA 3β-hydroxylase	Unpublished information
	GA2ox1	GA 2-oxidase ($GA_{1}/_{4}/_{9}/_{20}$ to $GA_{8}/_{34}/_{51}/_{29}$ and corresponding catabolistes)	Thomas et al., 1999
	GA2ox2	GA 2-oxidase	Thomas et al., 1999
	GA2ox3	GA 2-oxidase	Thomas et al., 1999
	GA2ox4	GA 2-oxidase	Thomas, Phillips and Hedden, unpublished
	GA2ox5	Probably pseudo gene	
	GA2ox6	GA 2-oxidase	Wooley, Phillips and Hedden, unpublished
	GA2ox7	GA 2-oxidase	Schomburg et al., 2003
	GA2ox8	GA 2-oxidase	Schomburg et al., 2003
C. maxima		K synthase	Yamaguchi et al., 1996
	CPS	CPP synthase	Smith et al., 1998
	GA7ox	7-oxidase (GA_{12}-ald./$_{14}$-ald. to $GA_{12}/_{14}$)	Lange, 1997
	GA20ox1	20-oxidase ($GA_{12}/_{53}$ to $GA_{25}/_{17}$)	Lange et al., 1994b; Lange, 1997
	GA20ox2	20-oxidase ($GA_{12}/_{14}/_{53}$ to $GA_{9}/_{4}/_{20}$)	Lange et al., unpublished
	GA3ox1	2β,3β-hydroxylase ($GA_{15}/_{24}/_{25}/_{17}$ to $GA_{37}/_{36}/_{13}/_{28}$)	Lange et al., 1997b
	GA3ox2	3-oxidase ($GA_{12}/_{15}/_{24}/_{25}/_{9}$ to $GA_{14}/_{37}/_{36}/_{13}/_{4}$)	Frisse et al., 2003
	GA3ox3	3-oxidase (GA_9 to GA_4)	Lange et al., unpublished
	GA2ox1	2-oxidase ($GA_{9}/_{4}/_{1}$ to $GA_{51}/_{34}/_{8}$)	Frisse et al., 2003

Developing pumpkin seeds are considered a rich source of GA biosynthetic enzymes (Graebe, 1987). Saito et al. (1995) purified *ent*-kaurene synthase (KS) from pumpkin endosperm, which was later cloned by Yamaguchi et al. (1996). The activity of CPS/KS (GGPP to *ent*-kaurene) was reported to be localized in wheat seedling developing chloroplasts and pumpkin endosperm leucoplasts (Aach et al., 1995). Pumpkin GA 7-oxidase has been isolated and expressed in *E. coli* (Lange et al., 1994a; Lange, 1997).

GA 20-oxidase1, a multifunctional enzyme, has been purified from pumpkin endosperm and its encoding cDNA was cloned (Lange, 1994; Lange et al., 1994b; Lange 1998). The pumpkin enzyme (CmGA20ox1) is responsible for the conversion of GA_{12} to GA_{15}, GA_{24}, and GA_{25}, as well as for the conversion of GA_{53} to GA_{44}, GA_{19}, and GA_{17}. The production of GA_{25} and GA_{17} is characteristic for pumpkin endosperm cell-free system (Lange et al., 1993b) and indicates that the 20-oxidase in this tissue is functionally different from those of other plant species (Kamiya and Graebe, 1983; Takahashi et al., 1986). Moreover, 20-oxidases were also cloned from various other plant species, e.g. *Arabidopsis* (Phillips et al., 1995; Xu et al., 1995), pea (Martin et al., 1996), tobacco (*Nicotiana tabacum*; Ueguchi-Tanaka et al., 1998), potato (*Solanum tuberosum*; Carrera et al., 2000), and hybrid aspen (*Populus tremula* X *Populus tremuloides*; Eriksson and Moritz, 2002).

GA 3-oxidases catalyse the final biosynthetic step to produce biological active GAs. 3β-hydroxylation results in the conversion of GA_{20} and GA_9 to GA_1 and GA_4, respectively. GA 3-oxidase genes have been found in several species, including *Arabidopsis* (Chiang et al., 1995; Williams et al., 1998; Yamaguchi et al., 1998), lettuce (*Lactuca*; Toyomasu et al., 1998), pea (*Pisum*; Lester et al., 1997; Martin et al., 1997), pumpkin (*Cucurbita*; Lange et al., 1997b), tobacco (*Nicotiana*; Itoh et al., 1999), and tomato (*Lycopersicon*; Rebers et al., 1999). The pumpkin GA 3-oxidase1 (CmGA3ox1) converts GA_{15} to GA_{37} (Lange et al., 1994a). This GA 3-oxidase1 (formerly called 2β,3β-hydroxylase) is a bi-functional enzyme which catalyses both, 2- and 3-oxidation, and prefers C_{20}-GAs as substrate (Lange et al., 1997b).

The GA 7-oxidase from pumpkin has not been found in other plant species. Phylogentically, pumpkin GA 20-oxidase1, and GA 20-oxidase from *Marah macrocarpus* are closely related to each other; they sequences share 67% identical amino acids (Figure 3). The pumpkin GA 3-oxidase1 (CmGA3ox1) amino acid sequence is related to the *Arabidopsis* GA 3-oxidase (GA4) at amino acid identity level by only about 35%, which is typical of dioxygenases with different functions (Prescott and John, 1996). Sequences of watermelon GA 3-oxidases are highly homologous to GA 3-oxidase1 (Kang et al., 2002). The pumpkin GA 2-oxidase1 (CmGA2ox1) shows highest amino acid similarity to a dioxygenase of unknown function previously cloned from *M. macrocarpus* (MacMillan et al., 1997; Frisse et al., 2003). Both share 84% identity, based upon their deduced amino acid sequences and, phylogenetically, both group with *Arabidopsis* GA 2-oxidase (Thomas et al., 1999).

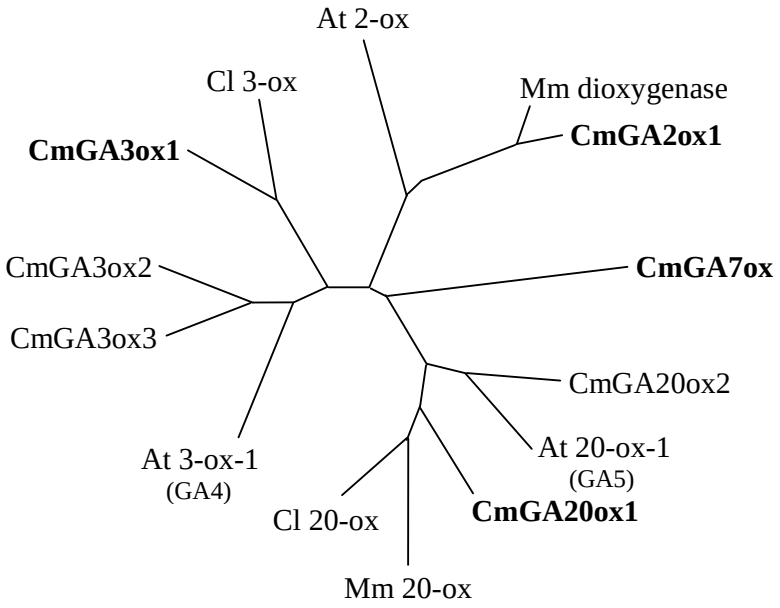


Figure 3: Phylogenetic analysis of deduced amino acid structures of selected GA oxidases from diverse species (Frisse et al., 2003). GA 7-oxidase from pumpkin (CmGA7ox); GA 20-oxidase from *Citrullus lanatus* (Cl 20-ox), *Marah macrocarpus* (Mm 20-ox), pumpkin (CmGA20ox2) and (CmGA20ox1), and *Arabidopsis* (At 20-ox-1); GA 3-oxidases from *C. lanatus* (Cl 3-ox), pumpkin (CmGA3ox1), (CmGA3ox2) and (CmGA3ox3), and *Arabidopsis* (At 3-ox-1); GA 2-oxidase from pumpkin (CmGA2ox1), and *Arabidopsis* (At 2-ox), and a dioxygenase of unknown function from *M. macrocarpus* (Mm dioxygenase).

1.4. Genetic modification of GA metabolism

The control of plant growth can be achieved chemically by using growth regulators. Many crops and plants have been treated with a range of chemical growth promoters, e.g. GA_3, which increase berry size (Christadoulou et al., 1968), and promote panicle elongation (Hedden and Hoad, 1994), or with chemical retardants that act by impeding different enzymes in the GA biosynthetic pathway. Recently, genetic manipulation of biosynthetic genes responsible for GA production provided an alternative approach in order to controlling plant growth.

The over-expression of the genes encoding for enzymes that catalyse the early stages of GA biosynthesis, e.g. *ent*-copalyl pyrophosphate synthase (*At*CPS) and *ent*-kaurene synthase (*At*KS) in *Arabidopsis*, showed no significant increased levels of bioactive GA and no effect on growth and plant development (Sun and Kamiya, 1994; Fleet et al., 2003).

GA 20-oxidase, a high regulatory enzyme, has been investigated intensively in genetic manipulation of GA biosynthetic pathway (Hedden et al., 1998). Huang et al. (1998) and Coles et al. (1999) reported that over-expression of *Arabidopsis* GA 20-oxidase in transgenic *Arabidopsis* plants resulted in the elongation of seedling hypocotyls, increased shoot growth, induced early flowering, and increased GA_4 level. Similarly, the over-expression of *Arabidopsis* GA 20-oxidase gene in hybrid aspen (Eriksson et al., 2000) and over-expression of the same gene from citrus or *Arabidopsis* in tobacco plants (Vidal et al., 2001; Biemelt et al., 2004) showed an increased level of bioactive GA and elongated phenotypes. Over-expression of GA 3-oxidase in hybrid aspen and *Arabidopsis* showed no significantly different in the morphology of transgenic plants (Israelsson et al., 2004; Phillips, 2004).

Recently, it has been shown that the so-called green revolution genes are involved in the GA signalling and biosynthesis (Peng et al., 1999; Spielmeyer et al., 2002; Monna et al., 2002; Sazaki et al., 2002). This approach offers an alternative strategy to introduce beneficial traits, such as dwarfism into cereal varieties to improve grain yield.

Another approach to control plant stature by genetic manipulation was the reduction of the level of bioactive GA. For example, antisense expression of GA 20-oxidase in

Arabidopsis (Coles et al., 1999) showed reduced stem elongation, delayed flowering and reduction of bioactive GA level. Carrera et al. (2000) showed that the expression of a *GA20ox* gene using antisense mRNA in potato reduced stem elongation and petiole length, and increased tuberization, with an increased tubers yield. Transgenic rice expressing antisense copies of OsGA3ox2 (*D18*) showed semi-dwarf phenotypes (Itoh et al., 2002). In addition, the ectopic expression of pumpkin GA 20-oxidase1 that produces mainly inactive GA products might produce a reduction of bioactive GAs by switching the pathway to the tricarboxylic acids and show dwarf phenotype. Although, the over-expression of *CmGA20ox1* in *Arabidopsis* resulted in the expected large increase in C-20 carboxylic acid GAs, the stem height of the transgenic plants were only slightly reduced (Xu et al., 1999). The same approach in *Solanum dulcamara* resulted in the accumulation of GA_{17} and semi-dwarfed plants (Curtis et al., 2000). In lettuce, dwarfed plants and a reduction of the levels of active GA (GA_1) were obtained by over-expression of *CmGA20ox1* under a very strong promoter cassette (Niki et al., 2001).

Another possibility to reduce endogenous GA level is to increase the rate of catabolic products by over-expression of GA 2-oxidases. The first cDNA encoding GA 2-oxidase was isolated from runner bean (*Phaseolus coccineus*) by a functional screening method (Thomas et al., 1999). Sequence information for 2-oxidases is available from a number of other species such as *Arabidopsis* (Thomas et al., 1999), pea (Lester et al., 1999; Martin et al., 1999), and rice (*Oryza sativa*; Sakamoto et al., 2001). Ectopic expression of GA 2-oxidase (*OsGA2ox1*) gene in rice resulted in a decrease of stem growth, small, dark green leaves, and destroyed development of the reproduction organs (Sakamoto et al., 2001). Whereas, expression of the same gene under the control of the shoot-specific *OsGA3ox2* promoter induced only semi-dwarfism without any negative effects on flower and grain development (Sakamoto et al., 2003). In addition, over-expression of GA 2-oxidase genes in *Arabidopsis*, tobacco and poplar showed severe dwarf phenotypes (Schomburg et al., 2003; Biemelt et al., 2004; Busov et al., 2003).

Over-expression of a runnery bean GA 2-oxidase in *Arabidopsis* and wheat resulted in a range of dwarf phenotypes, suggesting the superiority of this approach for the breeding of dwarf plants (Hedden and Phillips 2000b). In summary, the results

demonstrate that GA levels and, hence, plant growth and development, can be manipulated by genetic engineering of the genes of the GA biosynthetic pathway.

1.5. Aim of this work

The modification of plant stature is considered one of the most important requirements in agriculture, horticulture, and forest culture. This has been earlier achieved by plant breeding and the use of chemical plant growth regulators, which are exogenously applied to stimulate or retard elongation, often through chemical modification of GA biosynthesis. However, stature control through plant growth retardants requires repeated application of synthetic chemicals that is expensive, variable in effectiveness, and can have undesired environmental consequences or public perceptions. Therefore, biotechnological manipulation of GA levels provides an alternative approach that can be achieved through up- or down-regulating genes encoding enzymes involved in GA biosynthesis and catabolism.

The aim of this work is to manipulate the GA biosynthetic pathway through over-expression of GA-oxidases isolated from developing pumpkin seeds in *Arabidopsis thaliana*. We produced transgenic *Arabidopsis* expressing sense or antisense copies of pumpkin genes encoding GA-oxidases: 7-oxidase, 3-oxidase1, and 2-oxidase1 as well as sense copies of GA 20-oxidase1. *Arabidopsis* plants were transformed with the pumpkin genes downstream of a strong constitutive promoter cassette (E12-35S-Ω). The phenotypes of the transgenic plants were analysed, the expression levels of pumpkin GA-oxidase genes in *Arabidopsis* plants were determined by competitive RT-PCR, and the GA levels were quantified in transgenic plants to confirm to which extent GA biosynthesis is altered and to determine which steps of GA biosynthetic pathway are affected.

2. Material and Methods

2.1. Plant material and growth conditions

2.1.1. Plant material

All the work described was carried out using *Arabidopsis thaliana* ecotype Columbia provided from Botanischen Garten, Braunschweig.

2.1.2. Growth of plants in soil

Wild type (WT) seeds were sown onto the surface, of pre-wetted potting compost in Arasystem pots (http: //www. arasystem.com). The components of the potting compost are described below. The seeds were stratified at 4^{o}C for 2-3 days before transfer to a growth chamber, under long day conditions: 16 h light (105-120 µmol $m^{-2}s^{-1}$) and 8 h dark. The temperature was kept at 22^{o}C and 20^{o}C during the light and dark periods, respectively. *Arabidopsis* plants used for *Agrobacterium* mediated transformation were grown similarly but under relatively short day (13 h light, 11 h dark).

Components of potting compost (Compo SANA): 92% Peat
5% green compost
3% Perlite
Agrisol®
200-450 mg/l N_2
200-400 mg/l P_2O_5
300-500 mg/l K_2O
pH 5.5-6.5
Salt 1-2 g/l

Watering was performed regularly using tap water (5x time per week). Macronutrients (SUBSTRAL, Universal-DÜNGER) were supplied at least once a week for optimal growth. To obtain more floral buds per plant, inflorescences were clipped after most plants had formed primary bolts. Apical dominance was relieved encouraging synchronized emergence of multiple secondary inflorescences of about 1-10 cm tall (4-6 days after clipping).

2.1.3. Germination of plants in plates

For plate growth assays, seeds were sterilized as described below (2.1.5.) and germinated on 0.8% (w/v) plant agar in 0.5x Murashige and Skoog (MS) media including vitamins as described in Table 2 (Murashige and Skoog, 1962). MS salts and water were mixed by stirring, and the pH adjusted to 5.7 with KOH. Agar was added and the media sterilized by autoclaving.

Seeds germinate under long day conditions on MS plates. Plates were used for selection of transformants contain kanamycin (50 µg/ml) with or without 10^{-6} M GA_3. WT seeds were grown on MS media with or without 10^{-6} M GA_3. After growing on MS media for 2-4 weeks, the plants were transferred to soil.

Table 2: MS medium

Component	Amount to be added to make 1 liter
1x MS	4.33 g
plant agar	8 g
H_2O	to 1000 ml

When adding GA_3 to the medium:
GA_3 (346.4 mg) was dissolved in methanol 10^{-2} M stock solution and stored at -20°C. After sterilization, MS medium was allowed to cool to ~50°C; GA_3 was applied to a final concentration of 10^{-6} M.

2.1.4. Seed collection and storage

Plants were grown for 3-6 weeks until siliques were brown and dry. The inflorescence shoots from each pot were kept together and separated from those of neighbouring pots. Seeds were harvested by gentle pulling of grouped inflorescences through the fingers over a piece of clean paper. The majority of the stem and pod materials were removed by gentle blowing. Seeds were collected in envelopes and stored at room temperature.

2.1.5. Seed sterilization

Seeds were weighted (20 mg seeds) and put in an Eppendorf tube and 200 µl of 70% ethanol were added. Immediately 200 µl of H_2O were added and followed by 3x time rinses with double sterile water, then seeds were treated with 200 µl sodium hypochlorite (1.5%) for 5 min., followed by 3x times rinses with double sterilized H_2O and quickly air-dried on clean bench for 30 min.

2.2. Bacterial strain and plasmid

Agrobacterium tumefaciens strain has a C58C1 Rif^r (50 mg/l) chromosomal background and contained Ti plasmid pMP90-$Gent^r$ (20 mg/l). The *E. coli* bacterial strains and plasmids are listed in (Tables 3-5).

Table 3: The bacterial strains of *Escherichia coli*

Strain	Genotype	Reference
XL1-Blue	*recA*1, *endA*1, *gyrA*96, *thi*1, *hsdR*17 (r_k^-, m_k^+), *supE*44, *relA*1, λ^-, *lac*.[F′, *proAB*, *lacI*^{q}, *lacZ*ΔM15, Tn*10*(Tc^r)]	Bullock et al., (1987)
NM522	F′ *lac* I^qΔ(*lacZ*)M15 $proA^+B^+$/supE thiΔ (*lac* -proAB) Δ(hsdMS-mcrB)5 ($r_k^-m_k^-$ $McrBC^-$)	Gough and Murray, (1983)
HB 101	*supE*44 *hsd*S20($r_B^-m_B^-$) *recA*13 *ara*-14 *proA*2 *lacY*1 *gal*K2 *rps*L20 *xyl-5 mtl*-1	Boyer and Roulland, (1969) Boliver and Backman, (1979)

Table 4: Vectors

Plasmid	Genotype	Size (kb)	Reference
pBlueskript SK^-	Amp^r, *lacPOZ*′	2.95	Stratagene
pUC18	Amp^r, *lacPOZ*′	2.69	Hanna et al., (1984)
E12ΩMCS	Kan^r	11.5	Mitsuhara et al., (1996)

Table 5: plasmid Constructs

Plasmid name	DNA-fragments	Vector	Reference
7-oxidase (7ox)	CmGA7ox+Intron	pBlueskript	Frisse Andrea, (1999)
20-oxidase1 (20ox1)	CmGA20ox1+Intron	pBlueskript	Frisse Andrea, (1999)
3-oxidase1 (3ox1)	CmGA3ox1+Intron	pBlueskript	Frisse Andrea, (1999)
2-oxidase1 (2ox1)	CmGA2ox1-433 bp after digestion with HindIII	pBlueskript	Frisse Andrea, (1999)
7-oxidase sense	CmGA7ox sense	E12ΩMCS	Tomoya Niki, Japan
7-oxidase antisense	CmGA7ox antisense	E12ΩMCS	Tomoya Niki, Japan
20-oxidase1 sense	CmGA20ox1 sense	E12Ω	Niki et al., (2001)
3-oxidase1 sense	CmGA3ox1 sense	E12ΩMCS	Tomoya Niki, Japan
3-oxidase1 antisense	CmGA3ox1 antisense	E12ΩMCS	Tomoya Niki, Japan
2-oxidase1 sense	CmGA2ox1 sense	E12ΩMCS	Abeer Radi, this work
2-oxidase1 antisense	CmGA2ox1 antisense	E12ΩMCS	Abeer Radi, this work

2.3. Bacterial culture and growth conditions

2.3.1. Media

After preparation, all the media are autoclaved (Wolf Sanoclav; Shmidt, Braunschweig) for 20 min. Some components and antibiotics are added after autoclaving as indicated.

(LB) Luria-Bertani-Medium; Sambrook et al. (1989)

Trypton	10 g
Yeast extract	5 g
NaCl	5 g

Deionized water was added to approximately 1 liter. The pH was adjusted to 7.5 with 1 N NaOH. The final volume was adjusted to 1 liter and the media autoclaved (Wolf Sanoclav; Shmidt, Braunschweig) for 20 min. Solid LB medium was prepared by dissolving 35 g LB agar in 1 liter deionized water.

SOC-Medium

Trypton	4 g
Yeast extract	1 g
NaCl	0.1 g
KCl	0.04 g

The volume of solution was adjusted to 200 ml with deionized water and sterilized by autoclaving for 20 min.

Just before used, add:

$MgCl_2$, 1 M	2 ml
$MgSO_4$, 1 M	2 ml
Glucose, 2 M	2 ml

The $MgCl_2$ and $MgSO_4$ solutions were sterilized by autoclaving. Glucose solution was dissolved and sterilized by filtration through a 0.22-micro filter.

CPY-Medium; Sambrook et al. (1989)

Trypton	1 g
Yeast extract	0.2 g
Sucrose	1 g
$MgSO_4.7H_2O$	0.098 g

Deionised water was added to 200 ml. The pH was adjusted to 5.8 with 1 N NaOH and sterilized by autoclaving for 20 min. Solid CPY medium was prepared by adding 3 g agar.

2.3.2. Antibiotic

Stock solutions of antibiotics were prepared according to Sambrook et al. (1989), sterilized by filtration through a 0.22-micro filter, and then frozen at -20°C. The antibiotics were added to the autoclaved media cooled down to 50°C at the following final concentrations:

Carbenicillin	50 µg/ml in H_2O_{bidist}
Kanamycin	50 µg/ml in H_2O_{bidist}
Gentamycin	20 µg/ml in H_2O_{bidist}
Rifampicin	50 µg/ml in DMSO

2.3.3. Bacterial growth

A single bacterial colony containing the plasmid of interest was picked up from an agar plate and inoculated in LB-medium (*E. coli*) or CPY-medium (*Agrobacterium*) containing the appropriate antibiotic. The *E. coli* cultures were grown overnight (12-16 h) at 37^{o}C with vigorous shaking (Gesellschaft für labortechnik GmbH) and those of *A. tumefaciens* were grown for 2 days at 28^{o}C with vigorous shaking. Both bacterial cultures were grown to late log phase (i.e., to an OD_{600} of ~ 0.6). OD was determined by spectrophotometer at wavelength 600 nm in cuvette of 1 cm of liquid media.

2.3.4. Bacterial preservation

Bacteria can be stored for up to 2 years in cultures containing glycerol. In an Eppendorf tube containing 830 µl of bacterial culture (2.3.3.), 170 µl of 87% (w/v) glycerol (sterilized by autoclaving) was added. The mixture was vortexed to ensure that the glycerol is dispersed. The tube was placed for freezing in liquid nitrogen, and then transferred to -70^{o}C for long-term storage. To recover the bacteria, the frozen surface of culture was scraped with a sterile inoculating needle, and then the bacteria adhered to the needle immediately streaked on the surface of LB agar plate containing the appropriate antibiotic. *E. coli* plates were incubated (Memmert GmbH) overnight at 37^{o}C and *Agrobacterium* plates 48 h at 28^{o}C.

2.4. Nucleic acid preparation

2.4.1. Work with RNA and DNA

To obtain undegraded samples of RNA it is important that all equipment and solutions used in the preparation are free from ribonucleases (RNases). All solutions were prepared with RNase-free water and autoclaved for 20 min (Wolf Sanoclav; Shmidt, Braunschweig) 2x times prior to use. The glass tubes used were thoroughly cleaned and baked at 180^{o}C for 6 h before use. All other equipment (mortar, pestle, and spatulas) were soaked in 0.1% (w/v) SDS overnight and rinsed several times in double sterilized water prior to use. The pipette-tips used, were sterilized 2x times, and baked. As skin can be a potential source of RNase, gloves were used through out the preparation. The bench was cleaned with water and sprayed with ethanol 70%.

Diethyl pyrocarbonate (DEPC) was used to inactivate RNases that may contaminate solutions, glassware, and plastic-ware that are to be used for the preparation of nuclear RNA. Glassware was filled with a solution of 0.1% DEPC in H_2O were allowed to stand for one hour at 37^oC. The items were rinsed several times with DEPC-treated sterile distilled H_2O, and then autoclaved them for 15 min.

2.4.2. Phenol/chloroform extraction

For DNA-extraction, phenol/chloroform/isoamylalcohol 25:24:1 (v/v/v), pH 7.6 was used. For RNA-extraction, phenol/chloroform 5:1 (v/v), pH 4.7 was used.

For extraction of nucleic acids, one volume of the phenol/chloroform solution, then the extracts were mixed and centrifuged at 10.000 x g for 3 min at 4^oC (Sigma 3K30, Osterode) to separate two phases. The upper phase was removed and phenol/chloroform extraction performed two more times. At the end of the extraction, the upper layer was used for ethanol precipitation (2.4.3.).

2.4.3. Alcohol precipitation

For DNA precipitation, 1/10 volume of 7 M ammonium acetate was applied, followed by 0.6 volume of isopropanol. The mixture was incubated at 20.000 x g for 20 min at 4^oC, then centrifuged for 10 min at 4^oC. For RNA precipitation, 2.0-2.5 volumes of cold ethanol 100% were applied, and the mixture incubated for 1 h at -70^oC, and then centrifuged at 20.000 x g for 20 min at 4^oC, (Sigma 3K30, Osterode). In both cases, the pellet was washed with 70% ethanol and re-centrifuged for 10 min. The pellet was then dried at clean bench (Envirco, Ceag Schirp Reinraum technik; Bork) for 2-10 min, and then dissolved with 30-50 µl H_2O_{bidist}. The DNA solution was preserved at -20^oC while the RNA one was stored at -70^oC.

2.4.4. Determination of the concentration of nucleic acid by OD

The concentrations of DNA and RNA solutions were determined at the absorption wavelength (WL) 260 nm (OD_{260}) (Gene Quant II, Pharmacia Biotech; Cambridge). The absorption of 1.0 at 260 nm corresponded to 1 cm quartz cuvette (Hellma GmbH) was 50 µg/ml of double strand DNA (Davis et al. 1980), and was 40 µg/ml for RNA (Sambrook et al., 1989). The protein concentration was determined at WL 280 nm (OD_{280}). The OD_{260}/OD_{280} ratio was determined. The concentration of DNA or RNA

solutions were determined, analysed by agarose gel electrophoresis after staining with ethidium bromide, and photographed.

2.5. Isolation of nucleic acids

2.5.1. Isolation of genomic-DNA with CTAB

Fresh plant material (100 mg, two rosette leaves) were homogenized with pestle and mortar under liquid nitrogen, and then transferred to an Eppendorf tube. 150 µl CTAB-extraction buffer was added. After homogenisation with a micro-pestle, anther 500 µl CTAB-extraction buffer was added. The mixture was inverted several times and incubated for 30 min at 65°C in a water bath. The tubes were cooled down to room temperature and 600 µl chloroform was added. The mixture was several times inverted and centrifuged for 2 min at 10.000 x g (Biofuge Pico, Heraeus, Osterode) at room temperature. The aqueous phase was transferred to a fresh Eppendorf tube and 5 µl of RNase A solution (200 mg/µl) was added, incubated at room temperature for 30 min and precipitated with alcohol (2.4.3.). The pellet was dissolved with (20-100 µl H_2O_{bidist}), and stored at -20°C (Wilkie, 1996).

CTAB-extraction buffer:

Tris-HCl, pH 8.0	100 mM
EDTA	20 mM
CTAB (cetyltrimethylammonium bromide)	2%
NaCl	1.4 M
pH	8.0

2.5.2. Isolation of plasmid-DNA by mini preparation

From 10 ml of bacterial culture grown at 37°C, 700 µl was taken in 1.5 ml E-cup and centrifuged at 12.000 x g (Biofuge Pico, Heraeus, Osterode) at room temperature for 10 min. The pellet was re-suspended with 100 µl solution I (GETL-solution) and then 200 µl solution II (SDS-solution) was applied, mixed, and incubated for 3-5 min at room temperature. After that, 150 µl solution III (calcium-acetate-solution) was applied, the mixture chilled on ice bath for 10 min, and re-centrifuged at 20.000 x g for 10 min at 4°C. Supernatants were transferred in to fresh E-cup, followed by phenol/chloroform extraction (2.4.1.), and alcoholic precipitation (2.4.2.). The pellet

was dissolved in 40 µl H_2O_{bidist}. Plasmid-DNA was stored at -20°C (Birnboim and Doly 1979).

Solution I (GETL-solution):

Tris-HCl, (pH 8.0)	25 mM
Glucose	50 mM
EDTA	10 mM

Solution I was stored at -20°C. Before use immediately, add 0.5 mg/ml lysozyme.

Solution II (SDS-solution):

NaOH	200 mM
SDS	1% (w/v)

Solution III (calcium-acetate-solution):

Calcium acetate	3 M
Acetic acid	11.5% (v/v)

2.5.3. Isolation of plasmid-DNA by Qiagen Plasmid Midi Kit

A single colony from a freshly streaked *E. coli* selective plate was picked up and inoculated in 20 ml LB medium containing the selective antibiotic, and incubated at 37°C for 16 h with vigorous shaking ~300 rpm (Gesellschaft für labortechnik GmbH). The bacterial cells were harvested by centrifugation (6000 x g) for 15 min at 4°C (Sigma 3K30, Osterode). The bacterial pellet was re-suspended in 4 ml of buffer P1 and then 4 ml of buffer P2 added. The mixture was inverted 6x times, and incubated at room temperature for 5 min then, 4 ml of chilled buffer P3were added, mixed immediately but gently by inverting 6x times, and the mixture incubated on ice for 15 min and then centrifuged at 20.000 x g for 30 min at 4°C. The supernatant was placed in a fresh tube and re-centrifuged at 20.000 x g for 10 min at 4°C. A Qiagen-tip 100 was equilibrated by applying 4 ml QBT buffer by gravity flow. The supernatant was applied to the Qiagen-tip and allowed to enter the resin by gravity flow. The Qiagen-tip was washed by 2x 10 ml QC buffer and the DNA eluted with 5 ml QF buffer. Plasmid-DNA was precipitated by adding 3.5 ml (0.7 volumes) room temperature isopropanol, mixed, and then centrifuged immediately at 15.000 x g for 30 min at 4°C. The supernatant was carefully decanted. DNA pellet was washed with 2 ml of 70% ethanol at room temperature, and centrifuged at 15.000 x g for 10 min at 4°C. The

pellet was dried for 5-10 min under the clean bench (Envirco, Ceag Schirp Reinraum technik; Bork), and the DNA re-dissolved in 200 µl H_2O_{bidist}. Finally, the DNA concentration was determined (2.4.4.).

2.5.4. Isolation of total DNA from Agrobacterium

From 5 ml of *Agrobacterium* culture grown in CPY-medium, 1.5 ml was taken to a 2 ml E-cup and centrifuged at 6.500 rpm (Biofuge Pico, Heraeus, Osterode) for 1 min. Pellet was washed 2x times with 500 µl 100 mM Tris-HCl (pH 8.0) and re-centrifuged at 6.500 rpm for 1 min. The pellet was re-suspended in 600 µl *Agrobacterium*-DNA extraction buffer. Then, 50 µl Proteinase K (5 mg/ml) was added (without vortex), and 160 µl 10% SDS mixed gently, and then incubated for 2 h at 65^{o}C. The E-cup was cooled down at room temperature and 500 µl phenol/chloroform added. Separation of phases was taken place by centrifugation (15.000 rpm, for 20 min) and the DNA precipitated with 0.6-volume isopropanol, followed by 70% EtOH washing (2.4.3.). DNA-pellet was dissolved in 20 µl TE buffer.

Agrobacterium-DNA buffer (50 ml) pH (8.0):

Tris-HCl	110 mM
EDTA	55 mM
NaCl	1.54 M
CTAB	1.1%

TE-buffer (100 ml) pH (8.0):

EDTA	1 mM
Tris-HCl	10 mM

Tris-HCl, pH 8.0 (100ml):	100 mM
Proteinase K	5 mg/ml
SDS	10%

2.5.5. Isolation of RNA

2.5.5.1. Isolation of total RNA by Macherey-Nagel Kit

Plant tissue (50 mg) was ground with mortar and pestle (double sterilized) under liquid nitrogen. To lyse the cells, 350 µl RA1 buffer and 3.5 µl β-mercaptoethanol were added to the ground tissue and vortexed vigorously. NucleoSpin filter units were

placed in collecting tubes. The lysis mixture was applied, and centrifuged for 1 min at 11.000 x g (Biofuge Pico, Heraeus, Osterode). The filtrate was transferred to a new micro-centrifuge tube. To adjust RNA binding conditions, 350 µl ethanol (70%) was added to the recovered filtrate and mixed by vortex. For each preparation, one nucleoSpin-RNA plant column was taken in 2 ml centrifuge tube, the lysate loaded, and then centrifuged for 30 sec at 8.000 x g. The column was placed in a new collecting tube. Then, 350 µl of MDB (Membrane Desalting Buffer) was added and centrifuged at 11.000 x g for 1 min to dry the membrane. DNase reaction mixture was prepared in a sterile micro-centrifuge tube: for each isolation, 10 µl reconstituted DNase-I was applied to 90 µl DNase reaction buffer, and mixed by flicking. The DNase reaction mixture (95 µl) was applied directly onto the center of the silica membrane of the column and incubated at room temperature for 15-20 min. The nucleoSpin RNA plant column was washed by adding 200 µl RA2 buffer to inactivate the DNase and centrifuged for 30 sec at 8.000 x g. The column was re-washed by adding 600 µl RA3 buffer and re-centrifuged for 30 sec at 8.000 x g. After that, 250 µl of RA3 buffer was applied to the column, and centrifuged for 2 min at 11.000 x g to dry the membrane completely. The column was placed into a nuclease-free 1.5 ml micro-centrifuge tube. The RNA was eluted by adding 30-60 µl RNase-free water and centrifuged at 11.000 x g for 1 min. The RNA probe concentration was measured with a spectrophotometer (2.4.4.), The RNA quality was analysed by gel electrophoresis and aliquots were stored at -70^{o}C.

2.5.5.2. Treatment of RNA with DNase-I

To eliminate possible genomic DNA contaminations, it is always recommendable to treat the RNA samples with DNase I. The reaction mixture was prepared on ice to a final volume of 60 µl as follows:

RNA-Probe	50 µl
10x $MgCl_2$-buffer	6 µl
DTT 1 M (end concentration 10 mM)	0.5 µl
RNasin (5U)	0.5 µl
DNase I	1 U/µl reaction
H_2O_{bidist}	up to 60 µl

The reaction was incubated at 37°C for 20 min and stopped at -20°C. The probe (60 µl) was extracted with 3x times 150 µl phenol/chloroform 5:1 (pH 4.7) (2.4.2.), mixed and centrifuged. The upper phase was transferred in to a 1.5 ml Eppendorf tube with 10 µl 3 M sodium acetate (pH 5.5) and mixed. 250 µl (2.5 volume) cold ethanol (95-100%) was added. The probe was incubated 30 min at -20°C. RNA was precipitated by alcoholic precipitation (2.4.3.). The RNA pellet was dried on a clean bench and re-dissolved in RNase-free water (DEPC). The concentration of RNA was determined (2.4.4.) and the RNA sample frozen at -70°C.

2.6. Agarose-gel electrophoresis

A horizontal electrophoresis apparatus (GNA 100, Pharmacia Biotech etc. Midi-Wide Agagel, Biometra) with chamber, comb and a tape to form a mold was used. Electrophoresis buffer (always 1x TAE) was prepared to fill the electrophoresis tank. Agarose-gel was prepared at the concentration (0.8-1%) in electrophoresis buffer. The mixture was boiled in a microwave oven to dissolve the agarose. The agarose solution is cooled down and poured into the mold. Small amount of electrophoresis buffer was poured on the top of the gel, and carefully the comb and tape were removed. The probes were mixed with 0.20 volume of 6x gel-loading buffer. The samples migrated toward the positive (70 V for 90-120 min). The gel was stained by immersing it in electrophoresis buffer containing ethidium bromide for 10-15 min. The gel was washed for 5 min with H_2O_{bidist}, and UV photographed.

50x TAE-buffer:	2 M Tris-acetate 50 mM EDTA
6x loading buffer:	40% sucrose (w/v) in H_2O_{bidist} 0.25% bromophenol blue
Ethidium bromide staining:	0.5 µg ethidium bromide/ml in 1x TAE-buffer

2.6.1. DNA ladder and marker

The size of DNA fragments was determined by agarose gel electrophoresis (2.6.). To estimate the size of DNA fragments between 100-3000 bp, a 100 bp ladder was used

(MBI-Fermentas, St. Leon-Rot). For determination of DNA fragments up to 1500 bp, a *PstI* marker which was prepared by digestion of 40 µg λ-Phage DNA with PstI was used (2.7.1.).

PstI bands of λ-restriction fragments in bp (Sambrook et al. 1989):

14055[a], 11497, 5077, 4749, 4507, 2838, 2560, 2459, 2443, 2140, 1986, 1700, 1159, 1093, 805, 514, 468, 339, 264, 247, 216,210, 200, 164, 150, 94

([a]) Fragment can be compact together in the end of λ-*PstI*

2.7. Modification of DNA with enzymes

2.7.1. Digestion of plasmid DNA with restriction enzymes

The reaction mixture for the digestion of DNA with restriction endonuclease was prepared on ice as following:

Plasmid-DNA (1 µg)	x µl
10x reaction buffer	1 µl
Restriction endonuclease 2-10 U*	y µl
H_2O_{bidist} sterilized	up to 10 µl

*Not more than 10% glycerol of the final volume per µg DNA was used. All components were well mixed and incubated for 1-4 h or overnight at 37°C (Thermostat 5320, Eppendorf). The digestion was checked by agarose gel electrophoresis (2.6.). The digested plasmid DNA can be frozen at -20°C.

2.7.2. Dephosphorylation of DNA

Self-ligation of DNA fragments was prevented by dephosphorylation using Calf intestine Alkaline Phosphatase. Calf intestine alkaline phosphatase is an active enzyme, which removes the 5 prime phosphate group from the ends of DNA fragments. The reaction mixture for the dephosphorylation of DNA was prepared on ice as following:

DNA solution	10-40 µl
10x CIAP-reaction buffer	5 µl
Water, nuclease-free	to 49 µl

Calf Intestine Alkaline Phosphate 1 U	x µl

The reaction mixture was incubated at 37°C for 30 min and stopped by heating at 85°C for 15 min.

2.7.3. Purification of DNA with Cycle Pure Kit

This method was used to extract and purify DNA from an agarose gel. DNA samples mixed with gel-loading buffer were used to carry out electrophoresis. Agarose-gel was stained with ethidium bromide as described in (2.6.) and the DNA band of interest detected by UV. A sharp scalpel was used to cut out a slide of agarose containing the band of interest, which was then transferred to a clean 2 ml micro-centrifuge tube. After cut out the band, the gel was photographed to record that the DNA band of interest was removed. 4-5 volumes of CP-buffer was added for fragments <200 bp or 6 volumes of CP-buffer for fragments >4 Kb. 3 volumes CP-buffer plus 1 volume sterile water were applied. The top of the tube was closed, the gel melted by incubation for 10 min at 65°C, and the solution re-cooled at room temperature. The solution was transferred into 2 ml collecting filter tube, and centrifuged for 1 min at 10.000 x g (Biofuge Pico, Heraeus, Osterode) at room temperature. The filter was washed with 750 µl of DNA-wash buffer (buffer concentrate plus 1.5-volume absolute ethanol), and re-centrifuged for 1 min at 10.000 x g. The filter was dried by centrifugation for 1 min at 10.000 x g, the column replaced in a fresh Eppendorf tube, and the DNA eluted with 30 µl sterilized H_2O_{bidist}. The DNA can then be frozen at -20°C.

2.7.4. Ligation of DNA fragments

A 3:1 molar ratio of insert : vector DNA was used. The master mix was prepared on ice as following:

Linearized vector DNA	(0.2-1 µg)
Insert-DNA fragment at 3:1 molar ratio	x µl
10x ligation buffer	2 µl
PEG 4000 solution (for blunt ends only)	2 µl
Water, nuclease-free	up to 20 µl

T4 DNA ligase (1-2 U) for sticky ends, 5 U for blunt ends

The mixture was vortexed, spin down and then incubated for 1 h at 22°C or overnight at 15°C in a thermocycler (Progene; Techne, Cambridge). T4 DNA ligase was

inactivated by heating the reaction at 65°C for 10 min. The ligation was cleaned up with Cycle Pure Kit (Classic-Line; peqlab Biotechndogie GmbH, Erlangen) (2.7.3.). The resulting ligation reaction was used directly for bacterial transformation or after storing at -20°C.

2.8. DNA transfer

2.8.1. Transformation of Escherichia coli

2.8.1.1. Preparation of competent cells of E. coli

In a 50 ml Greiner tube, 10 ml LB medium was added and a single colony of *E. coli* XL1-blue inoculated. The culture was grown overnight at 37°C with shaking (Gesellschhaft für labortechnik GmbH). Then, 2.5 ml of the pre-culture was inoculated in 250 ml LB medium at 37°C with shaking (250 rpm) until an OD_{600} 0.5-0.7 is reached. The culture was transferred to ice for 5 min, divided in four tubes (4x 50 ml), and then centrifuged at 5000 x g for 5 min at 4°C (Sigma 3K30, Osterode). The pellet was washed with 60 ml double sterilized H_2O_{bidist}, re-centrifuged at 5000 x g for 5 min at 4°C, and rewashed 2x times with cold double sterilized H_2O_{bidist},. The pellet was suspended in 1.25 ml of 15% glycerol, and centrifuged at 5000 x g for 5 min. The pellet was re-suspended with cold 250 µl of 15% glycerol. Aliquots (40 µl) were frozen in liquid nitrogen and stored at -80°C until they come into use.

2.8.1.2. Transformation of E. coli by electroporation method

The frozen cells were placed on ice bath and immediately mixed with 2 µl plasmid DNA. The DNA was mixed with competent cells, the mixture transferred to a pre-chilled electroporation cuvette, and the following conditions for electroporation used:

Capacitance:	25 µF
Voltage:	2.5 kV
Resistance:	200 Ω
Pulse length:	5 msec

Immediately after electroporation, 960 µl of SOC medium was added to the cuvette. The mixture was transferred into a 15 ml Greiner tube and incubated at 37°C for 1 h with gentle agitation (Gesellschaft für labortechnik GmbH). The transformed cells

were plated (100-200 µl) in LB agar plate with antibiotic and incubated overnight at 37°C (Memmert GmbH).

2.8.2. Transformation of Agrobacterium tumefaciens

2.8.2.1. Preparation of Agrobacterium competent cells

Agrobacterium cultures harbouring Ti plasmids were grown at low temperature (28°C) to prevent "curing" of the Ti plasmid, on LB media containing antibiotics (Rif/Gent) for 24-48 h until an OD_{600} of 0.5-0.7 was reached. Cells were cooled on ice (20 min) and pelleted by centrifugation at 4°C for 15 min at 4.000 x g (Typnr. 3K30; Sigma). The media was completely poured off and the pellet re-suspended gently with cold double sterilized H_2O_{bidist} (50 ml). The cells were centrifuged and rewashed with cold water 3x times. The culture was washed with 1.5 ml of cold 10% (v/v) glycerol, re-centrifuged, and re-suspended in 250 µl of 10% glycerol. Aliquots (80 µl) were frozen in liquid nitrogen and could be stored at -70°C for at least 6 months.

2.8.2.2. Transformation of Agrobacterium by electroporation

The efficient transformation of *Agrobacterium* strain can be obtained by application of a high voltage electric pulse under conditions similar to those giving high frequency transformations of *E. coli* (Shen and Forde, 1989).

DNA for electroporation must be free of salt, RNA, or protein. Frozen competent cells were thawed on ice and the 80 µl aliquot was transferred to a pre-cooled 0.2 cm electroporation cuvette. 1 µl of of plasmid-DNA (2-10 ng) was mixed with the cell suspension on ice and an electric pulse was applied immediately using a Gene Pulser (E. coli Pulser, Bio Rad). The highest transformation efficiencies were obtained at:

Capacitance	25 µF
Voltage	2.5 kv
Resistance	200 Ω
Pulse length	8-12 msec

LB medium (1 ml) was immediately added to the 0.2 mm cuvette, the mixture transferred to a 15 ml Greiner tube and incubated for 3 h at 28°C with gentle agitation. Aliquots of 10 µl or 100 µl were plated on LB agar medium containing the appropriate antibiotics (Kan/Rif/Gent), and incubated for 2-3 days at 28°C.

2.8.2.3. Transformation of Agrobacterium by Tri-Parental Mating

Agrobacterium strain was inoculated into 3 ml CPY-medium and grown for 48 h at 28^{o}C with (Rif/Gent). Next day, both of the kan^{r} *E. coli* and a helper strain-*E. coli* HB 101 were inoculated in 3 ml LB medium and grown overnight at 37^{o}C (Gesellschaft für labortechnik GmbH). One day after, 0.5 ml kan^{r} *E. coli* cell culture, 0.5 ml helper strain-*E. coli* HB 101 cell culture, and 1 ml recipient cell culture (*Agrobacterium* strain) were mixed into the same tube. The mixtured cells were centrifuged for 5 min at 2.000 rpm (Sigma 3K30, Osterode), and the supernatant was removed. The pellet was re-suspended in 500 µl CPY, then plated on CPY agar plates without antibiotic and incubated at 28^{o}C for 48 h. The thin layer of bacterial cells was inoculated in 0.5 ml CPY and dilutions made in CPY medium until 10^{-6}. Mating mixture, (100 µl) of 10^{-4} and 10^{-6} dilutions were spread on CPY agar plates containing (Rif/Gent/Kan) and grown for 48 h at 28^{o}C. Small liquid cultures (3 ml) of the restreaked colonies were grown and minipreps carried out (2.5.4.) and /or polymerase chain reaction (PCR) were preformed to verify the presence of the plasmid DNA (2.9.2.)

2.9. Polymerase chain reaction (PCR)

2.9.1. (PCR)

By PCR (Polymerase Chain Reaction), a huge number of copies of a gene can be obtained. There are three major steps in a PCR (denaturation, annealing and extension), which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

In a sterile 0.5 ml PCR-cup kept on ice bath, the following reagents were mixed in a final volume of 10 µl:

10x PCR-buffer	1 µl
$MgCl_2$ buffer (25 mM)	0.6 µl
dNTPs (0.2 mM end concentration)	0.2 µl
Forward primer (10 pmol/µl)	0.2 µl
Reverse primer (10 pmol/µl)	0.2 µl
DNA-polymerase (5 U/µl)	0.06 µl

Template DNA (0.2-1 µg)	max 1 µl
H_2O_{bidist} sterilized	up to 10 µl

The nucleic acids were amplified in a thermocycler (Progene or Techgene; Techne, Cambridge) using one of the programs listed in Table 6. The primer used and their respective T_m value are listed in Table 7 and 8.

Table 6: PCR programs

No.	Program
1	3min 94°C+35x [30sec 94 °C+30sec 50 °C+2min 72°C]+5min 72 °C+99h 4°C
2	3min 94°C+35x [30sec 94 °C+2min 72°C]+5min 72 °C+99h 4°C
3	3min 94°C+35x [30sec 94 °C+30sec 60 °C+2min 72°C]+5min 72 °C+99h 4°C

Table 7: Primers for PCR, RT-PCR and bacterial screening

Name	Sequences 5´-3´	T_m-value*
7-ox For	TGGCTAACACAGGCATCCCTACTGTGGACG	71°C
7-ox Rev	TCTCATCCTCCACTCTTGAAGGTGGATGGG	69°C
RT 7-ox	ATTTCATAATGTTTGATGC	45.8°C
20-ox-1 For	TGAACGGCAAGGTGGCAACCGAATCCGCTC	72.2°C
20-ox-1 Rev	GCGGTAATAGTGGACCCAGTGTCCAACGCC	72.2°C
RT 20-ox-1	TTAAGCAGACGGGG	46.3°C
3-ox-1 For	ACAAAACGGTCTCGATCCCGGTTGTCG	68°C
3-ox-1 Rev	CCCTTCCCCGAGGCTTTAGCCTTGATG	69.5°C
RT 3-ox-1	TTGTTAGGGCAGCA	47.9°C
2-ox-1 For	CTCTGCAGCATTCTACTCTGGGATTCC	60°C
2-ox-1 Rev	GGCCCACCGAAGTAGATCATTGAAACC	60°C
RT 2-ox-1	AGATGTTGGAATCC	42°C

Table 8: Primer sequence for checking DNA integration by PCR

Name	Sequences 5´-3´	T_m-value*
Ω For (for all constructs)	CTACAACTACATCTAGAGG	47°C
20-ox-1 For (S orientation)	CGAGAATTCATAGAAATGATGGGC	60°C
20-ox-1 Rev (S orientation)	GACGAATTCCAGCAACACATAAGAC	61°C
7-ox Rev (S orientation)	TCACTCTAGAAGGTGGATGG	57°C
7-ox Rev (AS orientation)	CAATTCAAGGAAACGCTGG	54°C
3-ox-1 Rev (S orientation)	CGATTCTAGAAATATGCCATC	54°C
3-ox-1 Rev (AS orientation)	ACCGTTCTGTGATAAAGTGG	55°C

Calculation of annealing temperature:

*T_m= 69.3 + 0.41 x (GC %) – 650/(length of primers [bp])

2.9.2. Bacterial-colonies screening by PCR

A bacterial-colony was picked up from the growing plate using a sterile toothpick, touched it in master plate containing antibiotic and quickly washed the tip in 10 µl of PCR mixture (2.9.1.). In another method, a colony was picked up from the growing plate and washed in 10 µl of H_2O_{bidist} in an Eppendorf tube. The tube was closed and incubated in boiling water bath for 10 min (denature the templates and inactive proteases and nucleases). The tube was placed on ice and centrifuged for 2 min at 10.000 x g (Biofuge Pico, heraeus, Osterode). Then, 1 µl of DNA template was applied to the PCR-master mix (2.9.1.). These protocols were used for screening colonies of *E. coli* and *Agrobacterium*, and 10-20 colonies were checked.

2.9.3. Reverse transcriptase-PCR (RT-PCR)

For quantifying mRNA, a competitive RT-PCR with internal standard RNA was used. The RNA standards were added in a defined quantity prior to the reaction. The resulting standard cDNA was co-amplified with the same primers as the endogenous target sequence (Table 7). This method allowed measurement of small differences (as low as a factor of 2) between RNA samples. cDNA synthesis (PCR template) was generated in a double sterilized PCR-cup. The tubes were stored in ice water bath and used the protocol described below:

Total RNA (50-100 ng)	1 µl
Standard RNA* (100 pg to 1 fg)	1 µl
RT-primer (5 pmol/µl)	1 µl
Water, nuclease-free	up to 3 µl

The mixture was incubated at 70°C for 5 min and chilled on ice. The master mix was prepared as following components:

5x reaction buffer	1 µl
10 mM dNTP mix	0.5 µl
Ribonuclease Inhibitor (20 U/µl)	0.25 µl

The master mix was divided to make 1.75 µl in each PCR-cup and incubated at 37°C for 5 min and 0.25 µl of M-MULV (200 U/ µl) reverse transcriptase were added. The reaction mixture, containing the sequence-specific RT-primer, was incubated at 42°C for 1 h, stopped by heating at 70°C for 10 min, and chilled on ice. The synthesized cDNA was amplified by PCR (2.9.1.). Usually use 1 µl cDNA products as a template. The RNA standard was prepared as described in (2.9.4.) and the total RNA was isolated as described in (2.5.5.1.).

2.9.4. Generation of RNA-standards

Plasmid DNA of pblueskript SK⁻ vector containing the DNA of interest was linerized with a restriction endonuclease (2.7.1.). 7-oxidase was digested with restriction endonuclease (*Eco*RI), 20-oxidase1 (*Bam*HI), 3-oxidase1 (*Bam*HI) and 2-oxidase1 (*Kpn*I). The linerized DNA was purified with Cycle Pure Kit (2.7.3.), and checked by gel electrophphoresis (2.6.). The in vitro transcription of the DNAs of interest was performed using a T7 RNA promoter for 7-oxidase, 20-oxidase1, 3-oxidase1 and a T3 promoter for 2-oxidase1.In vitro transcription reaction was prepared as following:

5x transcription buffer	4 µl
10 mM NTPs mix	2 µl
linearized template DNA (1 µg)	x µl
Ribonuclease Inhibitor (1 U/µl)	0.5 µl
T3/T7 RNA Polymerase (40U)	2 µl
DEPC-treated water	up to 20 µl

The reaction was incubated at 37°C for 2 h and stopped by freezing at -20°C. The internal standard RNA was then treated with 1 U RNase-DNase-I and incubated at 37°C for 15 min to remove the plasmid DNA (the success of this treatment was checked by PCR without prior RT-PCR). The synthesized RNA was extracted with phenol/chloroform (2.4.2.), and precipitated with ethanol (2.4.3.). RNA standard concentration was quantitated by photometer (2.4.4.), diluted in 10 ng/µl, and frozen at -70°C. Standard concentrations are first added by a factor of 10 (1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, 0.1 pg/µl, 10 fg/µl, and 1 fg/µl) to determine the range in which the transcript amount is found. The main problem with RNA standards is their instability. We found that especially thawing and refreezing damages them. Therefore, we stored the standards in small aliquots in different dilutions and discard them if thawed too often. Standard RNA is analysed by agarose-gel electrophoresis (2.6.). Figure 4 showed the RNA standard analysed in agarose gel electrophoresis.

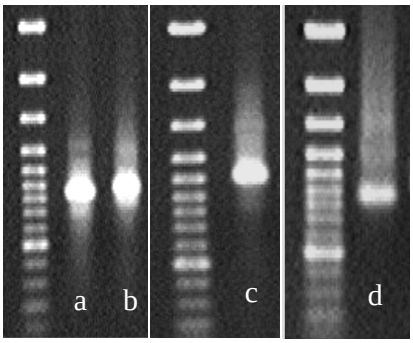


Figure 4: Analysis of RNA standard in agarose-gel (100 ng)

a: GA 3-oxidase1 RNA-standard b: GA 7-oxidase RNA-standard

c: GA 20-oxidase1 RNA-standard d: GA 2-oxidase1 RNA-standard

2.10. Culture of *Agrobacterium* and inoculation of plants

Agrobacterium tumefaciens strain C58C1 (PMP90), carrying the binary vector (E12-35S-Ω), was used in all experiments for which data are shown. Unless noted, bacteria were grown to stationary phase in liquid culture at 28°C at 250rpm in sterilized CPY-media (Sambrook et al., 1989) (2.3.1.) plus Kanamycin (50 µg/ml), Rifampicin (50 µg/ml) and Gentamycin (20 µg/ml). Three days prior to plant transformation, 5 ml of

liquid culture of *Agrobacterium* carrying the suitable binary vector was inoculated and incubated at 28^{o}C with vigorous agitation. After 2 days 400 ml of CPY-medium was inoculated with 1 ml of the pre-culture and incubated again with vigorous agitation for 24 h at 28^{o}C. The cells were harvested by centrifugation for 20 min at room temperature at 6000 x g when a final OD_{600} of approximately 0.80 was obtained. The pellet was then re-suspended in H_2O_{bidist} (100 ml). The revised floral dip inoculation medium (Clough and Bent, 1998) was used contained 5% sucrose and 0.05% (i.e. 500 µl) silwet L-77. For floral dip, the inoculums were added to sterile beaker, plants were inverted into this suspension such that all aboveground tissues were submerged, then removed after 1-2 min of gentle agitation. Dipped plants were removed from the beaker, placed in a plastic tray, and covered with a tall plastic dome to maintain humidity. Plants were left in a low light or dark location overnight and returned to growth chamber the next day. Plants were grown for a further 3-6 weeks until siliques developed. The inflorescences shoot from each pot were kept together and separated from neighbouring pots using the Arasystem (http: //www.arasystem.com).

2.11. Selection of transformants

After surface sterilization (2.1.5.), seeds of *Agrobacterium* inoculated plants, were suspended in 0.1% sterile agarose, spread on Kanamycin selection plates (0.5x MS media, 0.8% plant agar, and 50 µg/ml Kanamycin mono-sulphate) at a density of approximately 500 seeds per 150x 15 mm^2 plate. The plates were allowed to dry a little, so that the seeds do not float when the plate is moved. The seeds were cold-treated for 2 days at 4^{o}C and then grown for 2 weeks at 22^{o}C under 16h light/8h dark (100-110 µ Einsteins $m^{-2}s^{-1}$).

The excess moisture during growth was removed by opening the plates and removing moisture off the lid. Transformants were identified as Kanamycin resistant seedlings that produced green leaves and well-established roots within the selective medium. Gently the seedlings were pulled out of the agar and residual agar removed with a forceps. Roots were washed with sterilized H_2O_{bidist} before transferred to soil. Some transformants were grown to maturity by transplanting, preferably after the development of 3-5 adult leaves, into moistened potting soil. The transformation rates

were expressed as percentage transformation, and calculated as (# Kanamycin-resistant seedlings) / (total # seedlings tested) x 100.

2.12. Analysis of endogenous GAs

2.12.1. Extraction and purification of endogenous GAs

Plant material (2 g, wild type and transgenic) were harvested from 7-week-old seedlings grown in long day conditions. At harvest, the tissues were frozen in liquid nitrogen and ground with a mortar and pestle. The samples, approximately of 2 g fresh weight, were extracted with cold 80% (v/v) methanol (8 ml) at 4°C. After addition 2 ng of the internal standards ([$^2H_2$]GA_{12}-Ald., [$^2H_2$]GA_{12}, [$^2H_2$]GA_{24}, [$^2H_2$]GA_{15}, [$^2H_2$]GA_{44}, [$^2H_2$]GA_1, [$^2H_2$]GA_8, [$^2H_2$]GA_{20}, [$^2H_2$]GA_{19}, [$^2H_2$] GA_{53}, [$^2H_2$]GA_{34}, [$^2H_2$]GA_4, [$^2H_2$]GA_9, [$^2H_2$]GA_{17}, [$^2H_2$]GA_{25}, [$^2H_2$]GA_{14}, [$^2H_2$]GA_{36}, [$^2H_2$]GA_{37}), from Prof. L. Mandar, Australian National University, Canberra, Australia, the samples were mixed, incubated 1 h at 4°C with shaking and centrifuged 5 min at 3000 U/min (UJ2, Heraeus Christ GmbH, Osterode). The samples were re-washed 4x times with cold 80% methanol and incubated 30 min on ice during the extraction. The combined methanol extracts were concentrated in vacuo (Rotavapor RE 111; Büchi, Schweiz) and the resulting aqueous (aq.) residue was adjusted to pH 8.0 with KOH 1 M and extracted 3x times with ethyl acetate (ETOAc). The combined aq. phases were adjusted to pH 3.0 with HCl 1 N and extracted 3x times with ETOAc. The combined ETOAc phases were extracted 2x times with H_2O_{bidist} pH 3.2. The upper phases were collected and dried by evaporation in vacuo to give an acidic ETOAc-soluble fraction. The dried samples were stored at -20°C. Samples were methylated with 100 µl methanol and ethereal diazomethane (2x times 200 µl), and dried. Columns (C_{18}-cartridge (Waters) were washed with 10 ml MeOH and re-washed with 10 ml H_2O_{bidist} pH 3.2. Dry extractions were dissolved with 100 µl methanol and 2 ml acetic acid water pH 3.2 (HOAc). Probes were passed through the column. The glass tube of probe was re-washed with 2 ml HOAc, then passed through the column. Column was washed with 10 ml acetic acid water to remove proteins. The column was eluted with 6 ml of methanol (100%), and dried. The probes were dissolved in a small volume of 1:3 (v/v) methanol: H_2O_{bidist} pH 3.2. For purification by HPLC the residues were re-

dissolved in 200 µl methanol-water pH 3.2 (1:1) and injected into a C_{18} reverse-phase column (15 cm long, 8 mm, i.d., 4 mm, Novapack Liquid Chromatography Cartridge in a RCM100 radial compression system, Waters, Eschborn, Germany). Samples were eluted with a gradient from 25% methanol in water to 100% methanol in 40 min delivered by a two pump HPLC system (Model 501 and 510, Waters) at a flow rate of 1 ml min^{-1}. Eighteen fractions were collected at intervals from 11.50 to 47.50 min and fractionated by HPLC as the following program in Table 9:

Pump A: H_2O_{bidist} (pH 3.2): Methanol= 3:1

Pump B: Methanol

Time: 50 min **Cycle period**: 55 min

Table 9: Gradient program

Time (min)	Flow	%A	%B	%C	Curve
Initial	1.00	100	0	0	*
20	1.00	50	50	0	6
40	1.00	0	100	0	8
45	1.00	0	100	0	1
47	1.00	100	0	0	6

2.12.2. GC-MS analysis

Dried HPLC fractions were re-dissolved in (50 µl) 4x times methanol, dried, trimethylsilylated with 2 µl *N*-methyl-*N*-trimethyl-silyltrifluo-acetamid (MSTFA) and incubated for 30 min at 80°C. The derivatized samples were analysed using a Perkin Elmer TurboMass MS system (Perkin Elmer, USA) equipped with a Perkin Elmer AutoSystem XL gas chromatograph. Samples (1-2 µl) were co-injected into SGE BPX5 capillary column (30 m long, 0.25 mm i.d., 0.25 µm film thickness; SGE, U.K.) at an oven temperature of 60°C. The split value (30:1) was open after 1 min, and the temperature was increased by 45°C min^{-1} to 220°C and then with 4°C min^{-1} to 300°C. The He inlet was pneumatic pressure controlled at a constant flow rate of 1.5 ml min^{-1}

and the injector, transfer line and source temperatures were 220, 280, and 240^{o}C, respectively. Data were acquired in the SIM-mode after 5 min. The ions were monitored for quantification of endogenous GAs based on retention time and the co-occurrence of addition ions. Endogenous levels were calculated based on peak areas, with reference to known amounts of deuterated internal standards, and calibration curves for each compound. The concentrations of endogenous GAs were calculated by reference to calibration curves from the peak-area ratios of the following ion pairs: 506/508 (GA_1), 284/286 (GA_4), 207/209 (GA_{53}), 284/286 (GA_{25}), 239/241 (GA_{15}), 314/316 (GA_{24}), 270/272 (GA_9), 594/596 (GA_8), 207/209 (GA_{44}), 374/376 (GA_{19}), 418/420 (GA_{20}), 506/508 (GA_{34}), 492/494 (GA_{17}), 300/302 (GA_{12}), 270/272 (GA_{12}-aldehyde), 298/300 (GA_{14}), 284/286 (GA_{36}), 310/312 (GA_{37}).

2.13. Chemicals and enzymes

100bp-ladder marker	MBI-Fermentas, St. Leon-Rot
Acetone	Acros Organics, New Jersey
Acetic acid	Merck, Darmstadt
Agar	Sigma, Deisenhofen
Agarose	Biomol, Hamburg
Ammonium acetate	Sigma, Deisenhofen
β-Mercaptoethanol	Fluka chemic AG, Buchs
Bromophenol blue	Serva Feinbiochemica, Heidelberg
Calcium acetate	Merck, Darmstadt
Calf Intestine Alkaline Phosphatase	MBI-Fermentas, St. Leon-Rot
Carbenicillin	Biomol, Hamburg
Cetyltrimethyl ammonium bromide (CTAB)	Merck, Darmstadt
Chloroform ($CHCl_3$)	Merck, Darmstadt
Diethyl ether	Riedel-de Hoën, Seelze
Diethyl pyrocarbonate (DEPC)	Merck, Darmstadt
Dimethyl sulfoxide (DMSO)	Sigma, Deisenhofen
Dithiothreitol (DTT)	Biomol, Hamburg
DNA-polymerase	MBI-Fermentas, St. Leon-Rot
DNA-polymerase	Genecraft, Münster
DNase I	Sigma, Deisenhofen
dNTPs	MBI-Fermentas, St. Leon-Rot
Ethanol EtOH (CH_3CH_2OH)	Sigma, Deisenhofen
Ethidium bromide	Sigma, Deisenhofen
Ethyl acetate	Merck, Darmstadt
Ethylene diamine tetracetic acid (EDTA)	Merck, Darmstadt
GA_3	Sigma, Deisenhofen
Gentamycin sulphate	Sigma-Aldrich, Steinheim
Glucose	Merck, Darmstadt
Glycerin (glycerol)	Sigma, Deisenhofen
Helium	West fallen AG, Müster

Hydrochloric acid (HCl)	Sigma-Aldrich, Steinheim
Isopropanol	Merck, Darmstadt
Kanamycin mono-sulphate	Sigma-Aldrich, Steinheim
LB-medium	Sigma, Deisenhofen
Ligation puffer	MBI-Fermentas, St. Leon-Rot
Liquid Nitrogen	Linde, Höllriegelskreuth
Lysozyme	Biomol, Hamburg
Magnesium chloride ($MgCl_2$)	MBI-Fermentas, St. Leon-Rot
Magnesium sulfate ($MgSO_4$)	Merck, Darmstadt
Methanol MeOH (H_3COH)	Roth, Karlsruhe
MS-mineral salts medium with vitamins	Duchefa Haarlem, Netherlands
N-methyl-N-nitrosu-p-toluolsulphonamid	Merck, Darmstadt
N-methyl-N-trimethylsilytrifluoacetamid (MSTFA)	Macherey-Nagel-Düren, Germany
NTPs	MBI-Fermentas, St. Leon-Rot
PEG 4000 solution	MBI-Fermentas, St. Leon-Rot
Phenol (pH 4.3)	Sigma, Deisenhofen
Phenol/chloroform/isoamylalcohol	Biomol, Hamburg
Plant agar	Duchefa Haarlem, Netherlands
Potassium chloride (KCl)	Fluka chemic AG, Buchs
Potassium hydroxide (KOH)	Merck, Darmstadt
Primer	Metabion GmbH or Biomers.net
Proteinase K	Merck, Darmstadt
Restriction endonuclease	MBI-Fermentas, St. Leon-Rot
Restriction buffer (10x)	MBI-Fermentas, St. Leon-Rot
Reverse transcriptase with 5x reaction buffer	MBI-Fermentas, St. Leon-Rot
Rifampicin	Serva Feinbiochemica, Heidelberg
RNase A	Sigma, Deisenhofen
RNase-inhibitor (RNasin)	MBI-Fermentas, St. Leon-Rot
RNA-polymerase buffer (5x)	MBI-Fermentas, St. Leon-Rot
Silwet L-77	LEHLE SEEDS
Sodium chloride NaCl)	Roth, Karlsruhe

Sodium dodecyl sulfate (SDS)	Serva Feinbiochemica, Heidelberg
Sodium hydroxide (NaOH)	Merck, Darmstadt
Sodium hypochlorite (NaOCl)	Fluka chemic AG, Buchs
Sucrose	Fluka chemic AG, Buchs
T3/T7-RNA-polymerase with 5x transcription buffer (40U/µl)	MBI-Fermentas, St. Leon-Rot
T4-DNA-ligase	MBI-Fermentas, St. Leon-Rot
Tris	Biosolve LTD, Volkensswaard/NL
Trypton	Roth, Karlsruhe
Yeast extract	Roth, Karlsruhe

3. Experiments and Results

Gibberellins (GAs) are involved in the regulation of many aspects during plant development. To investigate the effect of altered GA levels on growth, transgenic *Arabidopsis thaliana* plants have been engineered to express either sense and antisense copies of GA 7-oxidase gene (*CmGA7ox*), 3-oxidase1 gene (*CmGA3ox1*) and 2-oxidase1 gene (*CmGA2ox1*) or sense copies of 20-oxidase1 gene (*CmGA20ox1*) from developing seeds of pumpkin under a strong constitutive promoter cassette E12-35S-Ω (Niki et al., 2001). The presence of the transgene was detected in the transformed *Arabidopsis* plants by polymerase chain reaction in the T_2 generation. The transgenic *Arabidopsis* plants were segregated three to one in the T_2 generation. Manipulation of gibberellin biosynthesis by expression of pumpkin GA-oxidase genes in the sense orientation led to an altered growth and plant development in *Arabidopsis* (chapter 3.1.). The expression levels of pumpkin GA-oxidase genes in *Arabidopsis* were determined by competitive RT-PCR (chapter 3.2.). The effect of altered GA biosynthesis by expression of pumpkin GA-oxidase genes on *Arabidopsis* GA content, were determined by combined gas chromatography-mass spectrometry (GC-MS) (chapter 3.3.).

3.1. Over-expression of pumpkin GA-oxidase genes and generation of transgenic lines

3.1.1. Preparation of transformation constructs

A strong promoter cassette containing a translational enhancer (E12-35S-Ω) was used to enhance the expression of pumpkin GA-oxidases (Figure 5). The construct was prepared by replacing the β-glucuronidase gene of pBI121 with the pumpkin GA 20-oxidase1 (*CmGA20ox1*) cDNA sequence in sense orientation as a XbaI-SacI fragment, and the CaMV 35S promoter sequence with the strong constitutive promoter cassette E12-35S-Ω from pBE2113 as a HindIII-XbaI fragment (Mitsuhara et al., 1996). The construct carrying GA 7-oxidase (*CmGA7ox*) in sense and antisense orientation, GA 3-oxidase1 (*CmGA3ox1*) in sense and antisense orientation was prepared by first replacing the β-glucuronidase gene of pBI121 with a synthetic DNA multiple cloning

site (MCS) which has unique restriction endonuclease sites. GA 7-oxidase and GA 3-oxidase1 were then inserted in sense and antisense orientation at the EcoRI site (Niki and Masaji).

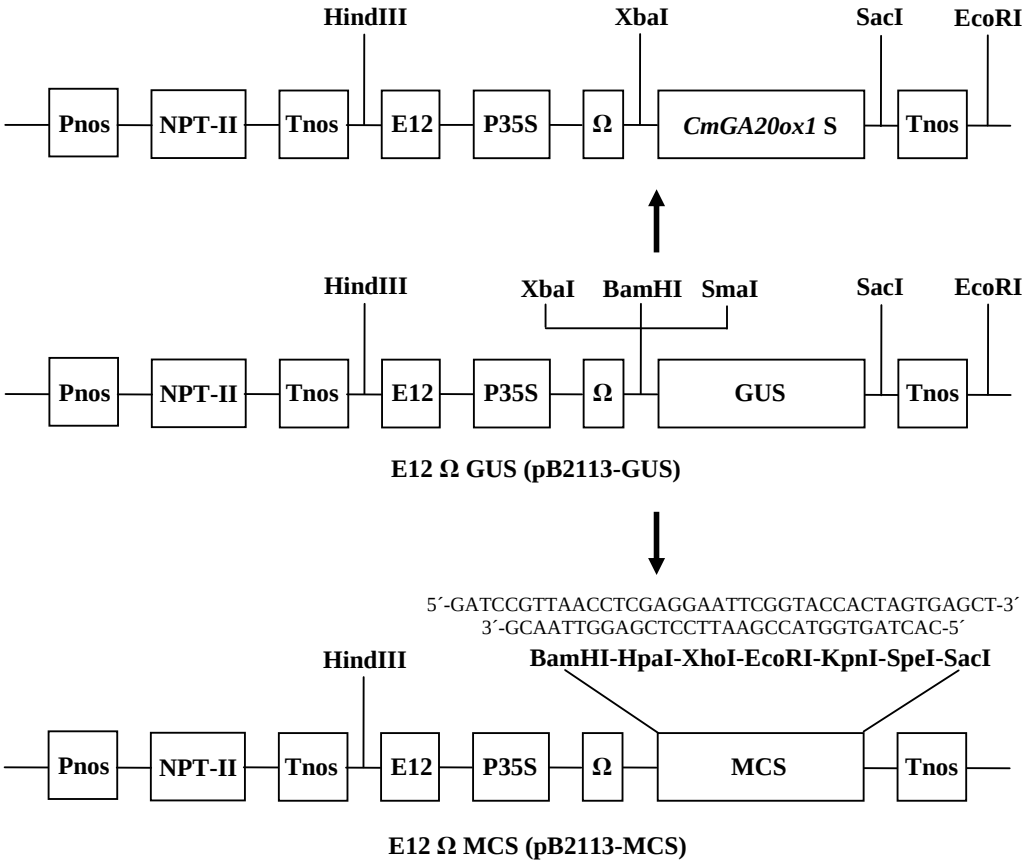


Figure 5: Structure of T-DNA region of pSGΩ (Niki et al., 2001). Pnos, 5´-upstream region of nopaline synthase gene. NPT-II, Coding region of nopaline synthase gene. Tnos, Polyadenylation region of nopaline synthase gene. E12, 5´-upstream region of CaMV 35S promoter (-419 to -90) X 2. P35S, 5´-upstream region of CaMV promoter (-90 to -1). Ω, 5´ -untranslated region of tobacco mosaic virus.

The pUC18 plasmid containing the cDNA insert of clone 2-oxidase1 (*CmGA2ox1*) (Frisse et al., 2003) was isolated (2.5.3.) and digested with EcoRI (2.7.1.). The E12-35S-Ω MCS vector was digested with EcoRI at the same site and dephosphorylated (2.7.2.). The 2-oxidase1 cDNA fragment and the vector were ligated (2.7.4.). The plasmid DNA containing 2-oxidase1 was transformed into XL1-blue competent cells by electroporation (2.8.1.2.) and the bacterial colonies screened by using PCR (2.9.2.).

The resulted PCR products were analysed by agarose gel electrophoresis (Figure 6a and b). The 13 colonies that gave positive result by PCR were grown overnight at 37^{o}C and their plasmid DNA were isolated by the mini preparation method (2.5.2.).

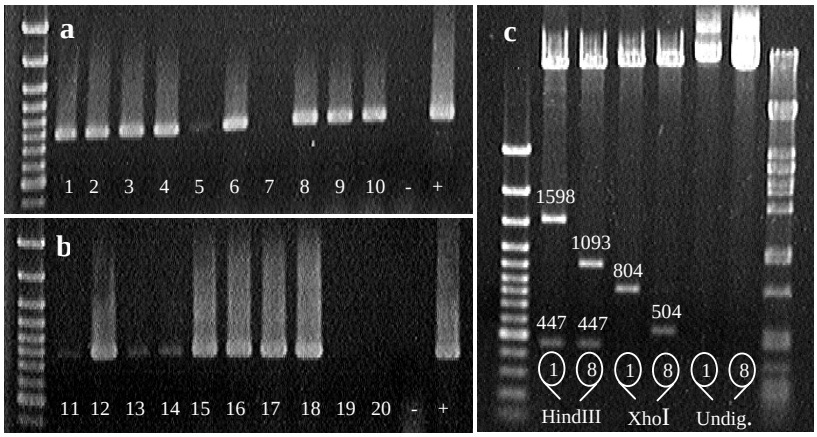


Figure 6: a, b: Colony screening by PCR, - negative control, + positive control (plasmid DNA of 2-oxidase1) and c: digestion of plasmid DNA from colony No. 1 and No. 8, with HindIII and XhoI to confirm the orientation of the insert. Undig., undigested plasmid DNA.

Sense (S) and antisense (AS) orientations of the full-length cDNA insert encoding for pumpkin GA 2-oxidase1 were confirmed by restriction endonuclease digestion. Clone No. 1 (*CmGA2ox1* AS orientation) and No. 8 (*CmGA2ox1* S orientation) were chosen and digested with HindIII and XhoI (Figure 6c). HindIII cuts the 2-oxidase1 gene at two positions and the binary vector at one position, releasing three fragments of 447 bp, 1598 bp and 10.7 kb in the case of 2-oxidase1 AS orientation, while 447 bp, 1093 bp and 11.3 kb in the case of 2-oxidase1 S orientation. Whereas XhoI cut each, 2-oxidase1 gene and binary vector, at one position, releasing two fragments of 804 bp and 12 kb in the case of 2-oxidase1 AS orientation, while 504 bp and 12.3 kb in the case of 2-oxidase1 S orientation. The constructs were transformed into *Agrobacterium tumefaciens*, strain C58C1, carrying the virulence plasmid pMP90 by electroporation (2.8.2.2.), or tri-parental mating (2.8.2.3.) method. Ten *Agrobacterium* colonies obtained for each transformation with the sense or antisense constructs were checked by PCR using specific primers for each of the GA-oxidases (Figure 7).

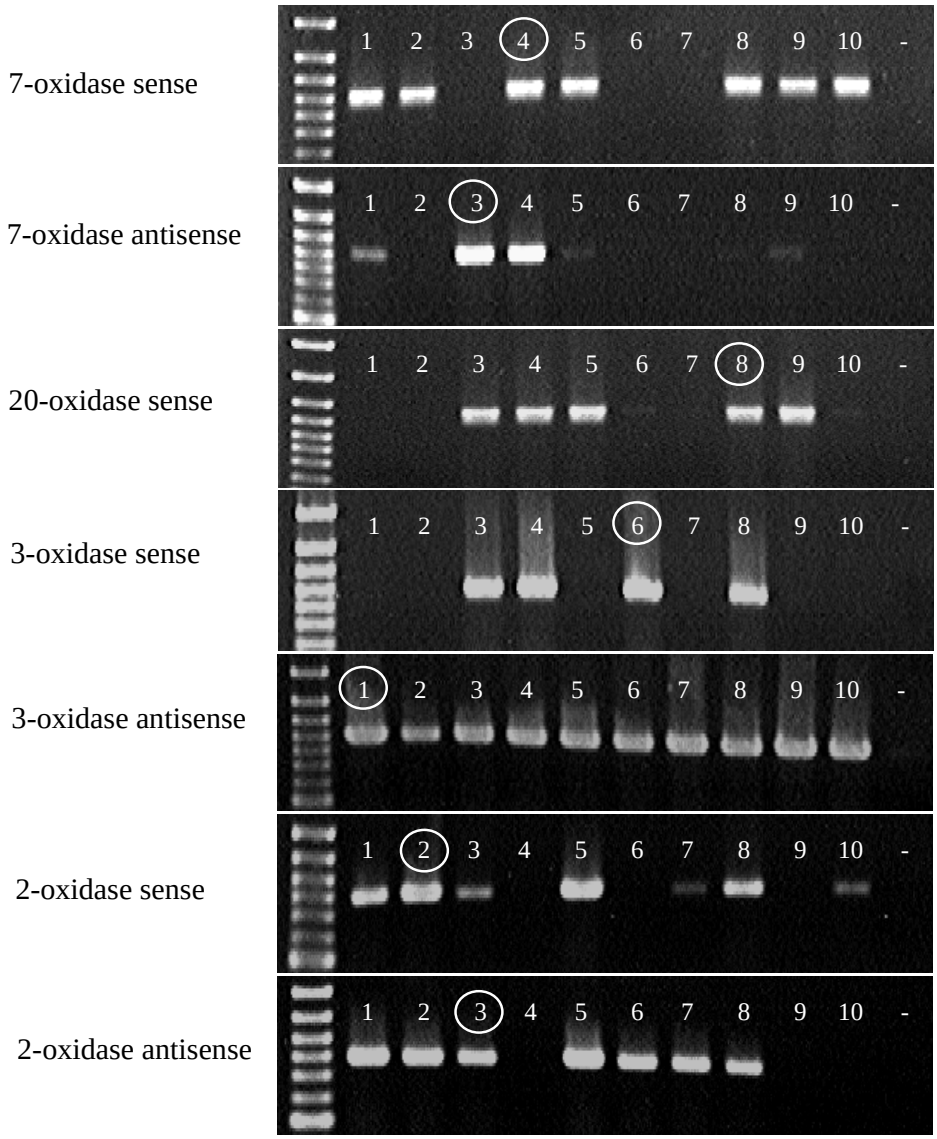


Figure 7: PCR screening of the *Agrobacterium* colonies transferred with constructs containing cDNAs of the pumpkin GA-oxidases in sense and antisense orientation. Circled number, colonies were chosen for plasmid DNA preparation to transform *Arabidopsis* plants. PCR products: 7-oxidase sense and antisense (7-ox For./7-ox Rev., 915 bp); 20-oxidase1 sense (20-ox-1 For./20-x-1 Rev., 1140 bp); 3-oxidase1 sense and antisense (3-ox-1 For./3-ox-1 Rev., 900 bp); 2-oxidase1 sense and antisense (2-ox For./2-ox Rev., 800 bp).

The *Agrobacterium* plasmid DNA for *Arabidopsis* transformation was checked by restriction digestion with (HindIII). HindIII cuts the 7-oxidase gene and the binary vector at one position, releasing two fragments of 1014 bp and 11.5 kb in the case of 7-

oxidase S orientation (Figure 8a), while 1845 bp and 10.7 kb fragments in the case of 7-oxidase AS orientation and cuts only the binary vector of 20-oxidase1 gene, releasing one fragment 12.7 kb (Figure 8a). With 3-oxidase1 (*CmGA3ox1*) and 2-oxidase1 (*CmGA2ox1*) genes, HindIII cuts each gene at two positions and the binary vector at one position, releasing three fragments: 503 bp, 961 bp and 11.3 kb in the case of 3-oxidase1 S orientation, while 503 bp, 1618 bp and 10.6 kb in the case of 3-oxidase1 AS orientation (Figure 8b); 447 bp, 1093 bp and 11.3 kb in the case of 2-oxidase1 S orientation, while 447 bp, 1598 bp and 10.7 kb in the case of 2-oxidase1 AS orientation (Figure 8c).

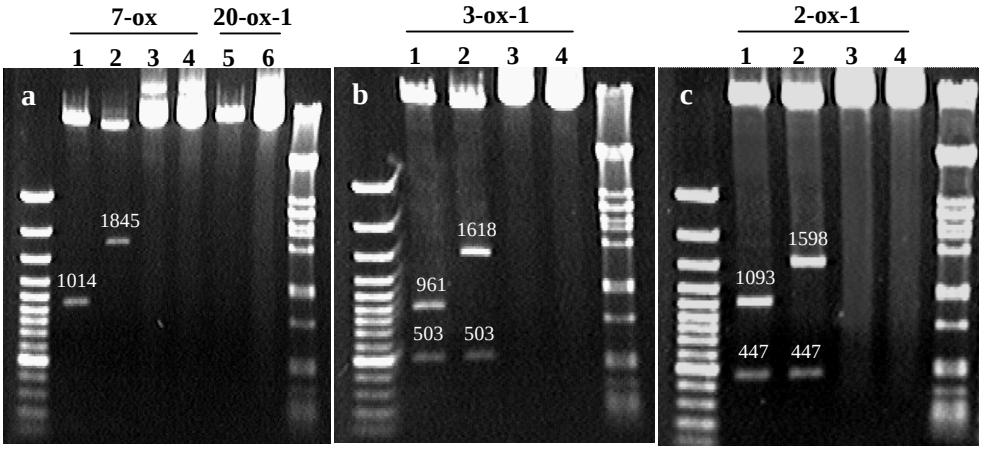


Figure 8: Agarose gel analysis of plasmid DNAs digested with HindIII.

1 and 5 sense digested with HindIII

2 antisense digested with HindIII

3 and 6 sense undigested

4 antisense undigested

3.1.2. Transformation and selection of Arabidopsis plants

Transformation of *Arabidopsis* wild type plant was performed by infection with *Agrobacterium tumefaciens*, harbouring the pumpkin GA-oxidases constructs, using the floral dip method (Clough and Bent, 1998) (2.10.). To get a high rate of transformation, *Arabidopsis* plants were dipped two times at seven-day intervals. The first dip was done after clipping and the second dip one week later. Ten thousand seeds for each construct were selected for kanamycin resistance. The rates of transformation

obtained by kanamycin selection were (0.7% for 7-oxidase S, 1.0% for 7-oxidase AS; 1.1% for 20-oxidase1 S; 0.9% for 3-oxidase1 S, 0.7% for 3-oxidase1 AS; 0.6% for 2-oxidase1 S, 1.0% for 2-oxidase1 AS). Twenty-five independent transgenic lines of three-week-old T_1 seedlings of each gene in sense and antisense were transferred to soil. The T_2 seeds from the self-fertilized flowers were sown on MS media containing kanamycin. Resistant to sensitive seedlings were segregated 3:1. The agreement fits to the theoretical 3:1 distribution predicted by mendelian inheritance. During the segregation analysis of *CmGA20ox1* S and *CmGA2ox1* S seeds, the germination exhibited a disturbed segregation ratio of T_2 generation seeds compared with wild type and antisense lines and the seeds had a high degree of dominance. The seeds exposed to apply GA_3 at 10^{-6}M during germination of seedlings for 4 weeks. The results show that both homozygous and heterozygous lines responded to GA_3 when compared either with WT plants, or AS lines. T_2 rosette leaves were collected, their genomic DNA extracted (2.5.1.) and the integration of the pumpkin GA-oxidases were tested by PCR using specific primers as illustrated in Figure 9. To confirm the orientation of GA-oxidase genes, we used Ω forward primer for all constructs. In the case of 7-oxidase sense (Ω/7-ox Rev. sense primer) or antisense (Ω/7-ox Rev. antisense primer), the PCR products were 950 bp, and 826 bp, respectively (Figure 9a). In 20-oxidase1 sense orientation (Ω/20-ox-1 Rev. sense primer) and (20-ox For./20-ox Rev.), the PCR products were 1350 bp, and 1001 bp, respectively (Figure 9b). In the case of 3-oxidase1 sense (Ω/3-ox-1 Rev. sense primer) or antisense (Ω/3-ox-1 Rev. antisense primer), the PCR products were 937 bp, and 808 bp, respectively (Figure 9c). In the case of 2-oxidase1 sense (Ω/2-ox Rev. sense primer) or antisense (Ω/2-ox Rev. antisense primer), the PCR products were 952 bp, and 1261 bp, respectively (Figure 9d).

Ten independent transgenic lines for sense orientation and five lines for antisense orientation were re-screened at T_3 generation to identify homozygous transgenic lines. Seedlings from the 7-oxidase transgenic plants (lines 8.9, 12.8, 13.1 for sense and 15.9, 14.2 for antisense), 20-oxidase1 transgenic plants (lines 10.8, 2.2, 17.2 for sense), 3-oxidase1 transgenic plants (lines 1.3, 19.4, 17.7 for sense and 6.1, 5.9 for antisense), and 2-oxidase1 transgenic plants (lines 5.5, 9.8, 12.9 for sense and 3.1, 7.7 for antisense) were cultured in a growth chamber and used to select homozygous

transgenic plants, which produced seeds 100% kan^r. All of the subsequent experiments were carried out with seeds from the homozygous lines of T_3 and T_4 generation.

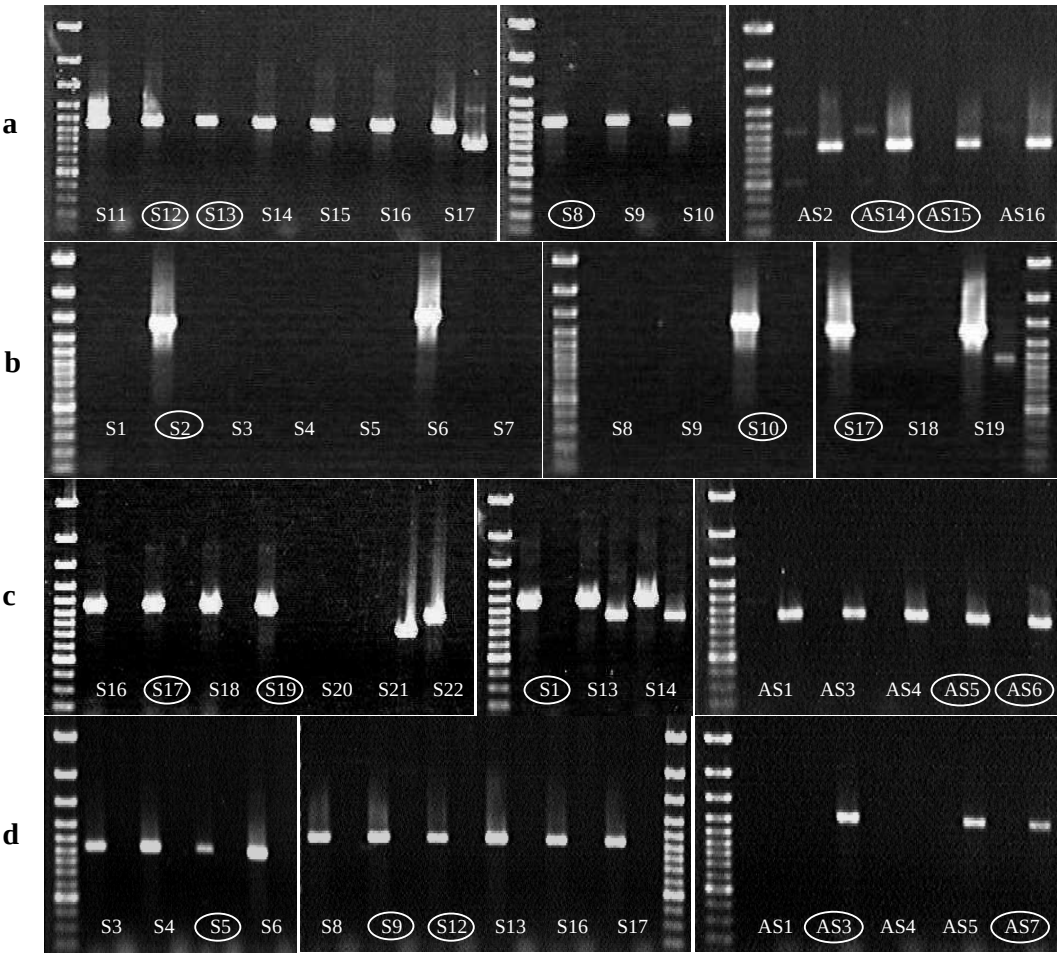


Figure 9: PCR analysis of the integration of pumpkin GA-oxidase genes by PCR. A circled numbers, the plants were chosen for segregation of homozygous lines.

a) 7-oxidase selection lines b) 20-oxidase1 selection lines

c) 3-oxidase1 selection lines d) 2-oxidase1 selection lines

3.1.3. Expression of GA-oxidases affect plant growth and morphology

To understand the importance of pumpkin GA-oxidase genes in plant development, we fused the full-length of 7-oxidase (*CmGA7ox*), 20-oxidase1 (*CmGA20ox1*), 3-oxidase1 (*CmGA3ox1*) and 2-oxidase1 (*CmGA2ox1*) cDNAs to E12-35S-Ω promoter cassette in sense and antisense orientation and introduced it into wild-type *Arabidopsis* plants by *Agrobacterium*-mediated gene transfer. We followed the development of two to nine-

week-old plants. *Arabidopsis* 14-day-old seedlings over-expressing *CmGA7ox* or *CmGA3ox1* showed altered root shapes when compared to wild type seedlings (Figure 10). The seedlings of *CmGA7ox* over-expression resulted in one thin long root with very few lateral roots while the seedlings of *CmGA3ox1* over-expression resulted in many thick lateral roots. Seedlings over-expressing *CmGA3ox1* showed also enlarged leaves and an increased number of trichomes relative to seedlings over-expressing *CmGA7ox* or wild type seedlings (Figure 10).

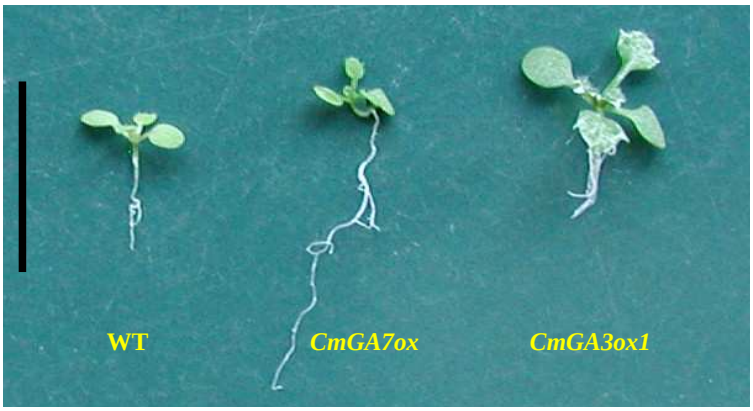


Figure 10: Phenotypes of 14-day-old seedlings gown in MS media. Wild type seedlings (left) compared to seedlings expressing sense copies of *CmGA7ox* (S12.8, middle) or expressing sense copies of *CmGA3ox1* (S17.7, right). Bar = 1 cm.

Most independent lines for *CmGA7ox* S and *CmGA3ox1* S for 7-week-old plants showed slender phenotypes (Table 10). Slender phenotypes are characterized by extremely rapid growth of seedlings and adult plant stages. The mature plants were tall, slender, and appeared to have constitutive GA responses. At the time of flowering, the homozygous plants were much taller than controls essentially because of increased internode length. The height of *CmGA7ox* S12.8 line for 7-week-old was increased relative to wild type or the two *CmGA7ox* AS14.2, AS15.9 lines. Similarly, the height of *CmGA3ox1* S17.7 line was extremely increased compared to wild type plants or plants transformed with antisense copies of *CmGA3ox1* lines AS6.1, and AS5.9 (Table 10). Their rosette leaf blades (nine-week-old) were pale green, slightly longer and wider than those of wild type plants and transgenic antisense lines (Figure 11, 12). *CmGA7ox* S12.8 line and *CmGA3ox1* S17.7 line flowered earlier (approximately 34

and 30 days after sowing, respectively) compared to control plants (Table 10). The internode length for *CmGA7ox* S12.8 line and *CmGA3ox1* S17.7 line of 7-week-old were approximately twice the length of either wild type plants or antisense lines.

Table 10: Phenotypic characterization of 7-week-old *Arabidopsis* plants over-expressing pumpkin GA-oxidases. Values are means of 5 plants/line ± standard deviation (SD).

Phenotype	Height of plant (cm)	Internode length (cm)	Number of Siliques	Flowering Time/day
WT	7.4 ± 1.0	1.2 ± 0.1	0.0 ± 0.0	42.6 ± 1.0
7-ox AS 14.2	8.4 ± 0.5	1.2 ± 0.1	0.0 ± 0.0	42.2 ± 0.8
7-ox AS 15.9	7.8 ± 0.5	1.2 ± 0.1	0.2 ± 0.4	41.4 ± 0.5
7-ox S 13.1	19.4 ± 1.1	2.0 ± 0.1	3.2 ± 1.6	39.8 ± 0.8
7-ox S 8.9	30.1 ± 0.7	2.1 ± 0.2	6.2 ± 1.8	36.0 ± 0.7
7-ox S 12.8	32.8 ± 3.1	2.1 ± 0.1	7.8 ± 1.5	34.2 ± 1.6
WT*	11.5 ± 0.7	1.4 ± 0.2	4.6 ± 2.1	41.2 ± 0.4
20-ox-1 S 10.8*	9.5 ± 0.6	1.2 ± 0.1	1.3 ± 0.8	40.0 ± 0.7
20-ox-1 S 2.2*	3.1 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	48.6 ± 0.5
20-ox-1 S 17.2*	1.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	48.6 ± 0.3
WT	8.6 ± 0.6	1.2 ± 0.1	0.0 ± 0.0	42.8 ± 1.1
3-ox-1 AS 6.1	7.2 ± 0.5	1.3 ± 0.1	0.0 ± 0.0	42.4 ± 1.1
3-ox-1 AS 5.9	8.0 ± 0.7	1.3 ± 0.1	1.0 ± 1.4	42.6 ± 0.5
3-ox-1 S 1.3	21.9 ± 1.3	2.0 ± 0.1	7.8 ± 0.8	32.4 ± 0.9
3-ox-1 S 19.4	28.9 ± 1.7	2.0 ± 0.1	17.6 ± 1.5	29.4 ± 1.1
3-ox-1 S 17.7	32.3 ± 3.1	2.1 ± 0.2	34.8 ± 1.3	30.0 ± 1.7
WT*	11.3 ± 0.9	1.5 ± 0.2	1.0 ± 1.4	41.0 ± 1.0
2-ox-1 AS 3.1*	10.8 ± 0.8	1.6 ± 0.1	1.0 ± 1.4	40.6 ± 1.3
2-ox-1 AS 7.7*	12.0 ± 0.4	1.6 ± 0.1	3.0 ± 1.2	42.0 ± 1.6
2-ox-1 S 9.7*	1.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	53.6 ± 1.0
2-ox-1 S 5.5*	1.3 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	54.8 ± 1.1
2-ox-1 S 12.9*	1.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	57.0 ± 1.0

* Plants have been transferred to soil after 28 days in MS media containing 10^{-6}M GA_3

The axes were slightly thinner and the number of siliques was much more in *CmGA3ox1* S than *CmGA7ox* S over-expressors (34.8 to 7.8). The phenotype of plants expressing antisense copies did not change compared to the wild type plants.

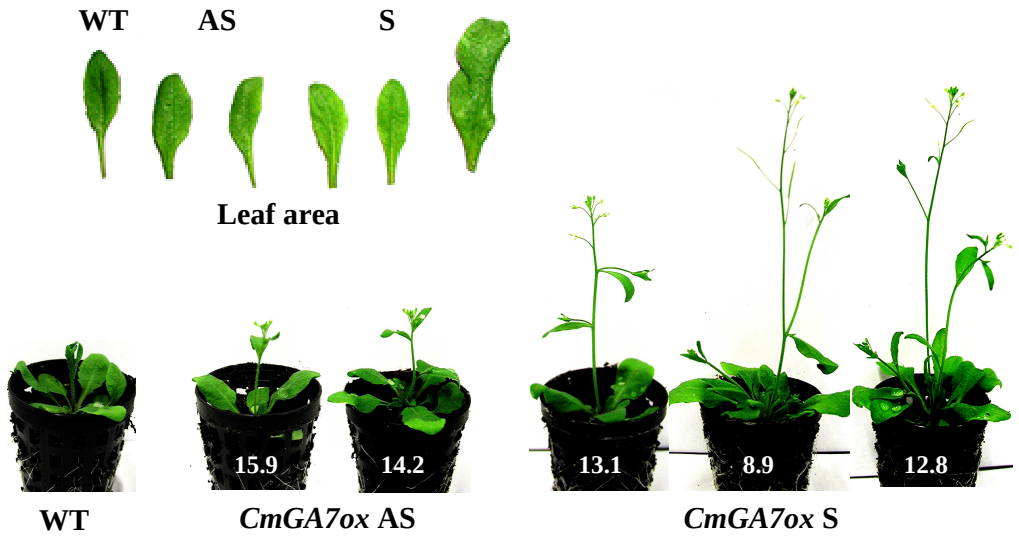


Figure 11: Morphological characterization of 7-week-old *Arabidopsis* plants containing sense (S) or antisense (AS) copies of pumpkin 7-oxidase (*CmGA7ox*). Wild-type plants (WT) and antisense lines are displayed as control.

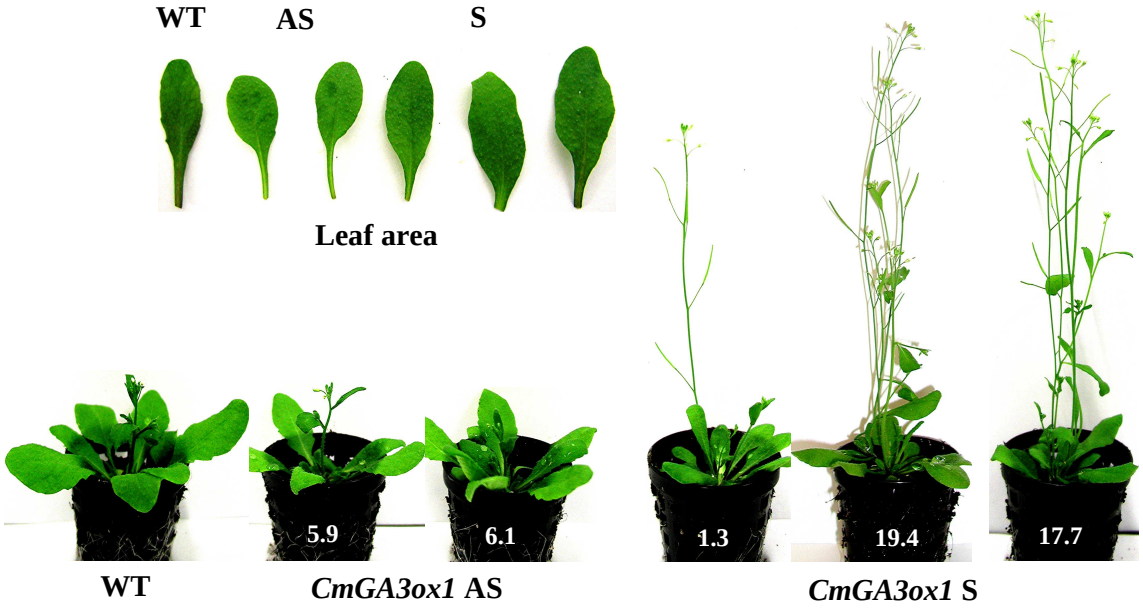


Figure 12: Morphological characterization of 7-week-old *Arabidopsis* plants containing sense (S) or antisense (AS) copies of pumpkin 3-oxidase1 (*CmGA3ox1*). Wild-type plants (WT) and antisense lines are displayed as control.

Most of transgenic lines (10 lines) obtained for *CmGA20ox1* S and *CmGA2ox1* S showed dwarf phenotypes (Figure 13 and 14). The transgenic *Arabidopsis* plants carrying *CmGA20ox1* S10.8, S2.2, and S17.2 lines showed a range of phenotypes. The height of the transformants ranged from 9.5 to 1.3 cm, whereas that of the wild type was 11.5 cm (Figure 13 and Table 10). The transgenic S17.2 line had a dwarf phenotype and showed the strongest expression of the *CmGA20ox1* as examined by RT-PCR (Figure 18). After the same number of days of vegetative growth, the size of the leaves of the *CmGA20ox1* over-expressors were considerably less than those of the control plants (Figure 13). These leaves had also shorter petioles and a slight bluish green colour compared to those of the control plants. In addition, there was a difference in the time of the flowering between the control plants and *CmGA20ox1* expressing lines. The control plants bolted and formed flowers well before the *CmGA20ox1* S expressing lines did (Table 10). The control plants (WT) began to bolt at 41 days, whereas the over-expressing lines S2.2 and S17.2 took 48 days. The *CmGA20ox1* S expressing plants went on to form flowers, siliques and set seed. The final seed yields were considerably less than that obtained with control plants. The lower yield of seed was due to, at least in part, the *CmGA20ox1* S17.2, and S2.2 lines producing fewer axillary shoots. The control plants had an average of seven leaves per rosette, whereas the *CmGA20ox1* over-expressing lines S10.8, S2.2, and S17.2 had 9, 6, and 12 leaves per rosette, respectively.

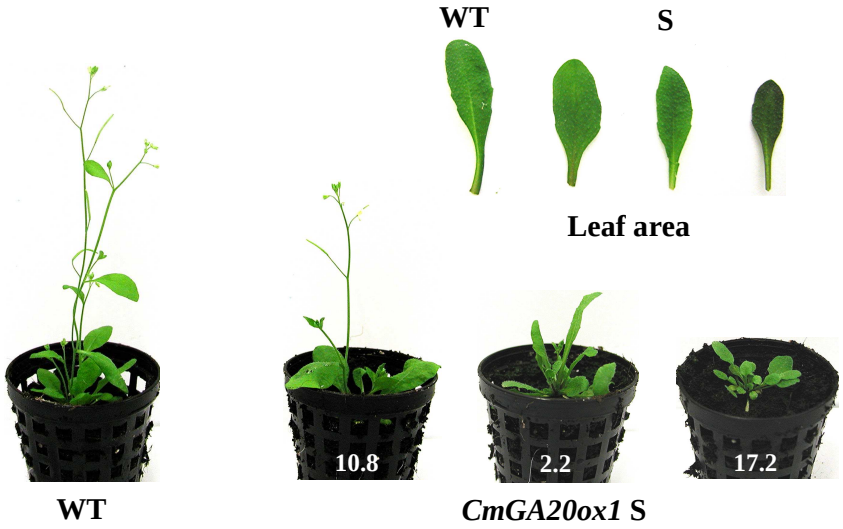


Figure 13: Morphological characterization of 7-week-old *Arabidopsis* plants containing sense (S) copies of pumpkin 20-oxidase1 (*CmGA20ox1*). Wild-type plants (WT) are displayed as control.

The *Arabidopsis* plants over-expressing the pumpkin 2-oxidase1 (*CmGA2ox1*) showed a typical phenotype for GA deficient dwarf plants (Figure 14). The plants were dwarfed with a strongly retarded phenotype. The dwarf line possess small and dark green rosette leaves compared to that of wild type and to the plants having copies of the *CmGA2ox1* in the antisense orientation (Figure 14). A range of dwarf phenotypes was observed for the *CmGA2ox1* over-expressors. The lines S9.7, S5.5, and S12.9 were chosen for further characterization. *CmGA2ox1* S12.9 line showed the most severe dwarf phenotype compared to S5.5 and S9.7 lines (Figure 14). It had a reduced height of 1.1 cm whereas the wild type had a height of 11.3 cm. In line S12.9, the flowering was delayed up to 2 weeks compared to antisense lines and wild type plants. Internode elongation in the extremely dwarfed transformants was not observed even with nine-week-old plants. The transgenic lines showing severely dwarf phenotypes did not bear any seeds after 7 weeks (Figure 14 and Table 10).

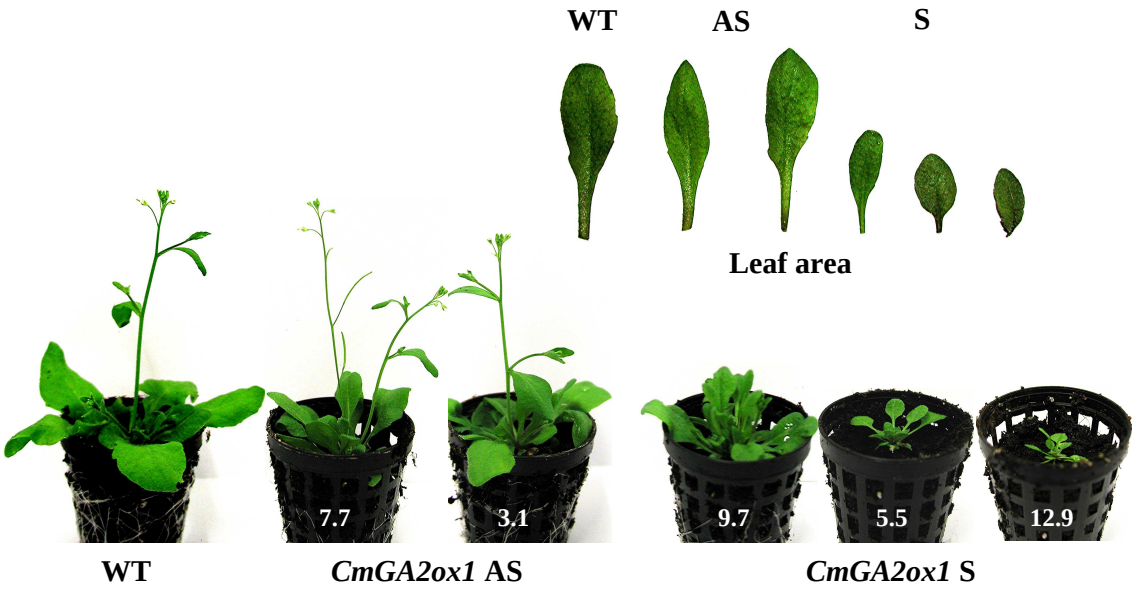


Figure 14: Morphological characterization of 7-week-old *Arabidopsis* plants containing sense (S) or antisense (AS) copies of pumpkin 2-oxidase1 (*CmGA2ox1*). Wild-type plants (WT) and antisense lines are displayed as control.

Arabidopsis plants over-expressing pumpkin 7-oxidase (*CmGA7ox*) and pumpkin 3-oxidase1 (*CmGA3ox1*) resulted in plant that develop more siliques in long day at week nine after sowing. The siliques of the *CmGA3ox1* over-expressors matured earlier than those of the *CmGA7ox* over-expressors. Figure 15 shows the effect of over-expressing *CmGA7ox* S12.8 line and *CmGA3ox1* S17.7 line on final seed weight per *Arabidopsis* plants. The final seed weight for *CmGA3ox1* sense over-expressors was significantly higher (2.6 g) than *CmGA7ox* sense over-expressors (1.7 g). *Arabidopsis* plants over-expressing pumpkin seed specific *CmGA20ox1* sense or *CmGA2ox1* sense had less siliques (Table 10). Figure 15 demonstrates that expression of both pumpkin *CmGA20ox1* sense and *CmGA2ox1* sense affect the yield of surviving seeds. The final seed weight observed with *CmGA20ox1* S17.2 line over-expressors was reduced to 0.6 g, while with *CmGA2ox1* S12.9 line over-expressors seed mass was reduced to 0.1 g. There was no significant different in seed weight between antisense and wild type plants.

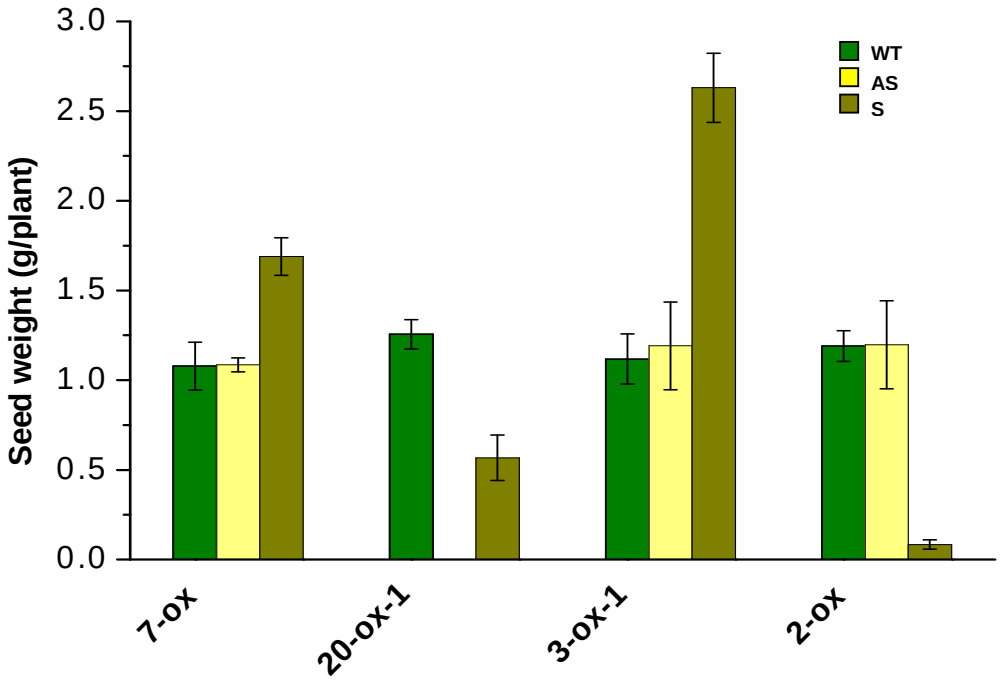


Figure 15: Seed weight of 9-week-old *Arabidopsis* plants expressing sense (S) or antisense (AS) copies of pumpkin *CmGA7ox*, *CmGA3ox1* and *CmGA2ox1* and sense copies of pumpkin *CmGA20ox1*. Wild-type plants (WT) of the respective developmental stage are displayed as control.

3.2. Quantification of pumpkin GA-oxidase expression by RT-PCR

3.2.1. Quantitative RT-PCR

RT-PCR (reverse transcription-polymerase chain reaction) is the most sensitive technique for mRNA detection and quantification currently available. RT-PCR can be used to quantify mRNA levels of small samples. In fact, this technique is sensitive enough to quantify RNA from a single cell (Prediger, 2001). Because of its sensitivity, RT-PCR has been coupled with other protocols for absolute quantification purposes. In competitive RT-PCR, known amounts of an internal standard are co-amplified in the same reaction tube with the sequence of interest, allowing the expression levels of the gene(s) under investigation to be determined (Freeman et al., 1999) (Figure 16).

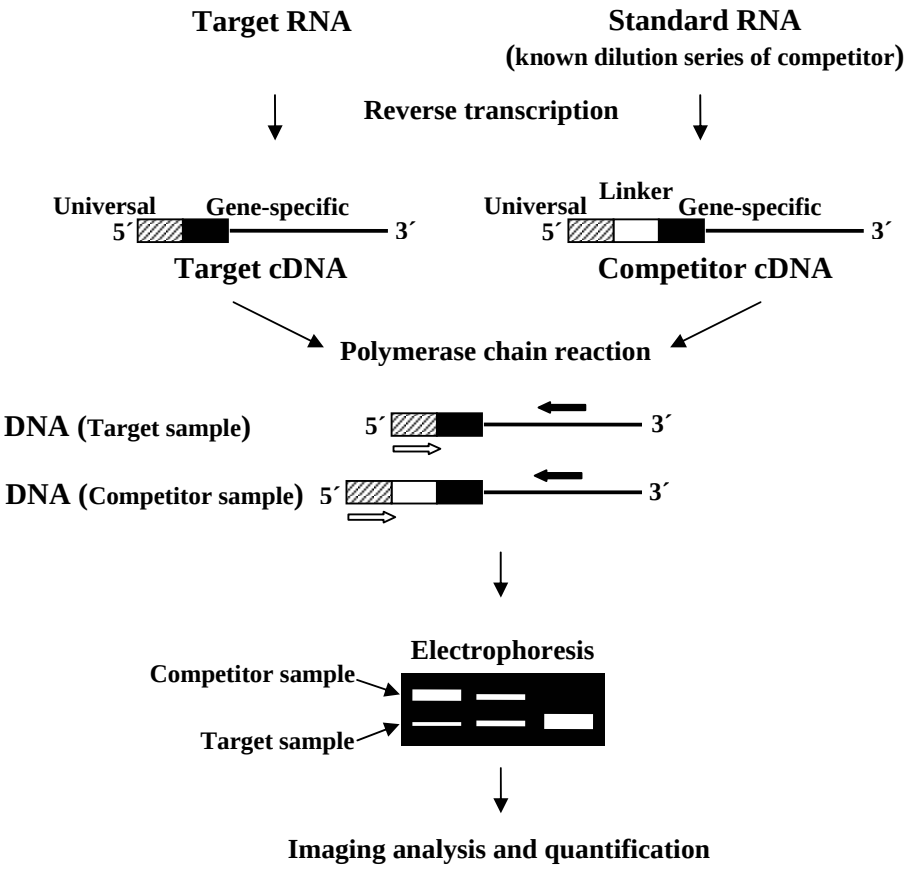


Figure 16: Diagram of quantitative RT-PCR.

The internal standard competes with the native sequence of the gene(s) of interest for primers, deoxynucleoside triphosphates, enzyme, and other reagents, thus reducing the signal of the native gene when the standard is in excess. As the amount of the internal standard increases, the signal of the native gene decreases. Co-amplifying an internal standard provides an efficient method of relating the product yield to the initial amount of transcript (Wong et al., 1994).

3.2.2. Quantification of the expression of pumpkin GA-oxidases in the transgenic Arabidopsis lines by RT-PCR

For the preparation of internal RNA standards, amplified genomic DNA of each GA 7-oxidase (*CmGA7ox*) and GA 20-oxidase1 (*CmGA20ox1*) genes was used containing ~200 bp-long introns. For the GA 3-oxidase1 (*CmGA3ox1*) gene, amplified genomic DNA containing ~150 bp-long introns was used (Frisse, 1999). For preparation of internal RNA standards for the GA 2-oxidase1 (*CmGA2ox1*), pBluescript SK plasmid was digested with HindIII that released a 448 bp fragment. The vector containing the remaining cDNA was re-ligated and used for standard RNA synthesis (Frisse et al., 2003). Plasmid-DNA was isolated from single transformants by using a Qiagen plasmid Midi Kit (2.5.3.). Plasmids (1 µg each) containing inserts in sense orientation coding for *CmGA7ox, CmGA20ox1* and *CmGA3ox1* were transcribed in vitro by using a T_7 transcription kit (MBI-Fermentas, St. Leon-Rot, Germany), whereas a T_3 transcription kit was used to transcript *CmGA2ox1* gene. RNA molecules were purified by using phenol/chloroform (2.4.2.), ethanol precipitated (2.4.3.), stored at -70^{o}C, and used as internal RNA standards.

Before performing quantitative RT-PCR, total RNA samples were analysed by agarose gel electrophoresis (2.6.) to ensure that the RNA was not degraded. For quantification, total RNA (50 ng) supplemented with different amounts of RNA standards and sequence-specific antisense RT primers (5 pmol) (Table 7) for each of the four genes were reverse transcribed using first-strand cDNA synthesis reactions in a total volume of 5 µl. One micro-liter of each of the reverse-transcribed products was amplified by PCR (2.9.1.) using sequence-specific sense (F) and antisense (R) primers (2 pmol each) (Table 7). Products were analysed by electrophoresis on 1% agarose gel, stained by ethidium bromide and visualized by UV translumination. Quantification of RNA

expression levels were done by comparing the intensity of the bands for each gene with those produced by internal RNA standards.

RNA expression levels for the four GA-oxidase genes were determined in pre-selected homozygous lines of *Arabidopsis* by RT-PCR. Transcript levels of pumpkin 7-oxidase were determined for the three sense lines (S13.1, S8.9, and S12.8) by comparison of *CmGA7ox* transcript (915 bp, lower bands) and different amounts of *CmGA7ox* standard (1100 bp, upper bands). They contained 60, 80, and 100 µg of transcripts per g of total RNA, respectively. No transcripts were found in the wild type plants and antisense lines. The difference in the phenotype correlated with the difference of 7-oxidase expression levels in the sense lines (Figure 17), plants having higher expression levels showed a more pronounced phenotype difference when compared to wild type plants or antisense lines (Figure 17).

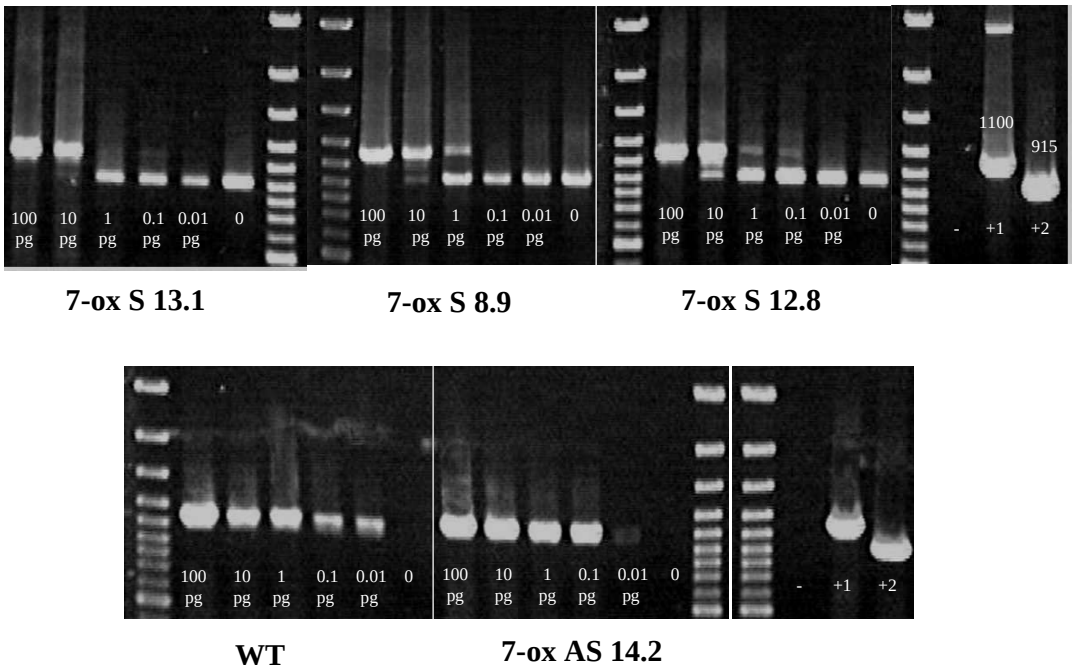
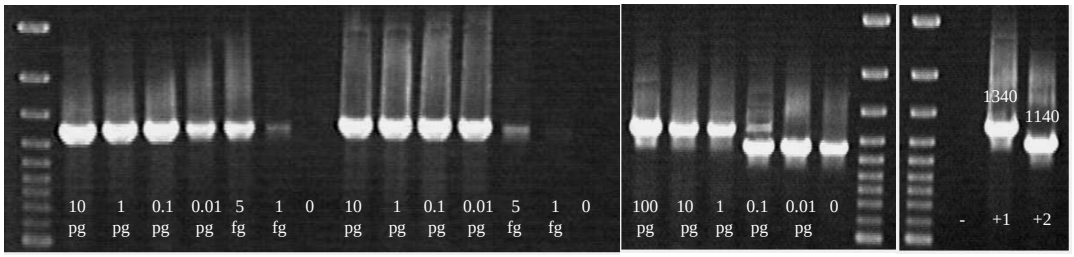
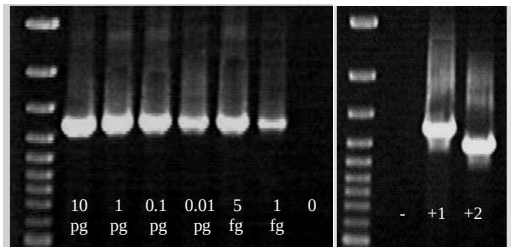


Figure 17: Expression levels of transgenic *Arabidopsis* plants containing sense (S) or antisense (AS) copies of pumpkin GA 7-oxidase (*CmGA7ox*) lines. The upper bands represent the standard (1100 bp); the lower are the transcript bands (915 bp). - negative control, +1 positive control (plasmid DNA of 7-oxidase plus intron), +2 positive control (plasmid DNA of 7-oxidase). 100 pg; 10 pg; 1 pg; 0.1 pg; 0.01 pg and 0 are the amounts of the RNA standard.

Transcript levels of *CmGA20ox1* were identified in only one line (S17.2) of the *Arabidopsis* plants transformed with sense copies of pumpkin GA 20-oxidase1. In line S17.2 was estimated to 10 µg of transcripts per g of total RNA by comparison of *CmGA20ox1* transcripts (1140 bp, lower bands) with *CmGA20ox1* standard (1340 bp, upper bands). In the other two transgenic lines S10.8, and S2.2 as well as in wild type plant no transcripts were detected by RT-PCR (Figure 18).



20-ox-1 S 10.8 **20-ox-1 S 2.2** **20-ox-1 S 17.2**



WT

Figure 18: Expression levels of transgenic *Arabidopsis* plants containing sense (S) copies of pumpkin GA 20-oxidase1 (*CmGA20ox1*) lines. The upper bands represent the standard (1340 bp); the lower are the transcript bands (1140 bp). - negative control, +1 positive control (plasmid DNA of 20-oxidase plus intron), +2 positive control (plasmid DNA of 20-oxidase). 100 pg; 10 pg; 1 pg; 0.1 pg; 0.01 pg; 5 fg; 1fg and 0 are the amounts of the RNA standard.

Transcript levels of *CmGA3ox1* were determined in three sense lines by comparison of *CmGA3ox1* transcript (900 bp, lower bands) with different amounts of *CmGA3ox1* standard (1051 bp, upper bands) and were calculated to be 20, 100, and 1000 µg of transcripts per g of total RNA for S1.3, S19.4, and S17.7 lines, respectively. No transcripts levels were detected in antisense lines or wild type plants (Figure 19).

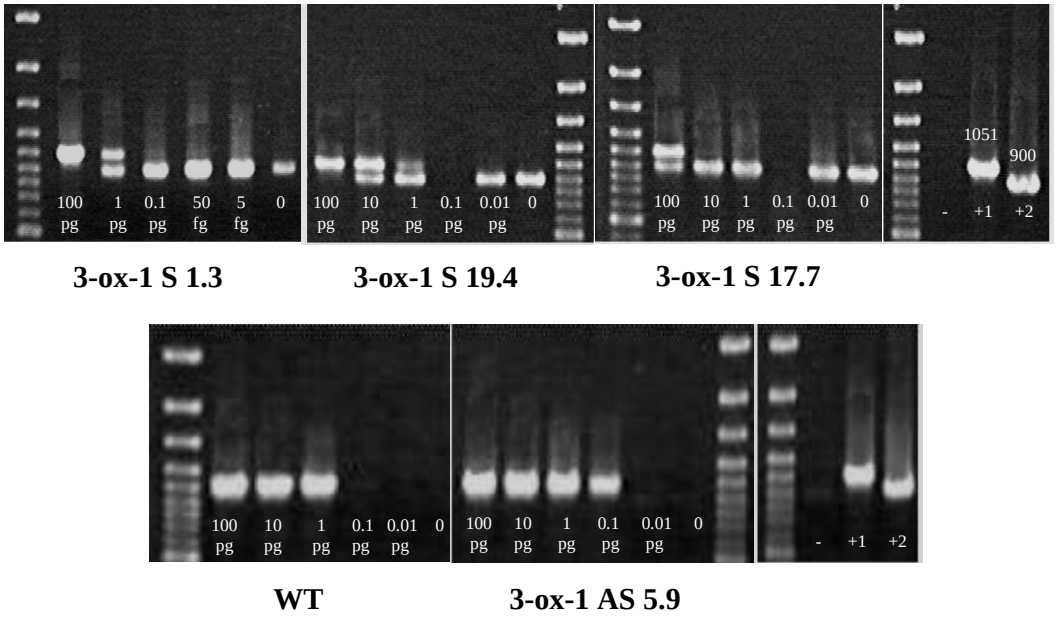


Figure 19: Expression levels of transgenic *Arabidopsis* plants containing sense (S) or antisense (AS) copies of pumpkin GA 3-oxidase1 (*CmGA3ox1*) lines. The upper bands represent the standard (1051 bp); the lower are the transcript bands (900 bp). - negative control, +1 positive control (plasmid DNA of 3-oxidase plus intron), +2 positive control (plasmid DNA of 3-oxidase). 100 pg; 10 pg; 1 pg; 0.1 pg; 50 fg; 0.01 pg; 5 fg and 0 are the amounts of the RNA standard.

CmGA2ox1 expression levels were estimated to be 20, 90 and 125 µg transcripts per g of total RNA for the three most dwarf lines S5.5, S9.8 and S12.9, respectively. No transcripts for the pumpkin GA 2-oxidase1 were detected in *CmGA2ox1* AS lines and WT plants. The difference in the phenotypic severity in the 2-oxidase sense lines was due to the different pumpkin GA 2-oxidase1 expression levels (Figure 20). Line S12.9 showed the highest expression level and the most severe dwarfed phenotype.

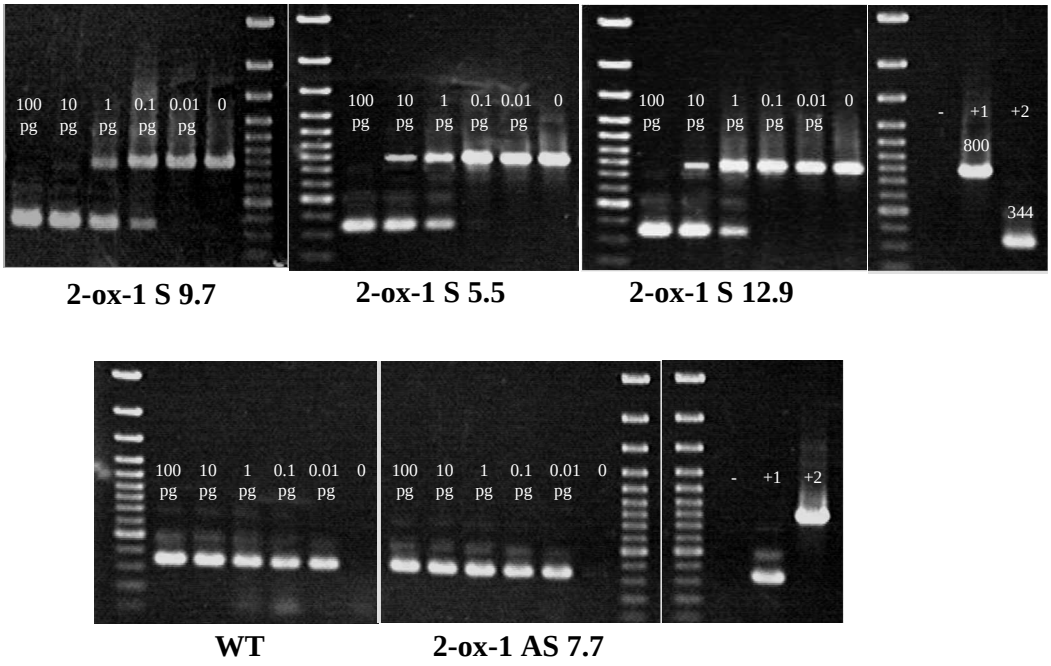


Figure 20: Expression levels of transgenic *Arabidopsis* plants containing sense (S) or antisense (AS) copies of pumpkin GA 2-oxidase1 (*CmGA2ox1*) lines. The upper bands represent the transcript (800 bp); the lower are the standard bands (344 bp). - negative control, +1 positive control (plasmid DNA of 2-oxidase), +2 positive control (plasmid DNA of 2-oxidase minus 448 bp fragment). 100 pg; 10 pg; 1 pg; 0.1 pg; 0.01 pg; and 0 are the amounts of the RNA standard.

3.3. Quantification of endogenous GA levels in transgenic lines

We analysed endogenous GA levels in 7-week-old transgenic *Arabidopsis* plants (shoot part) by gas chromatography-mass spectrometry to analyse whether GA biosynthesis is altered in the phenotype of transgenic lines and to determine to which extent and which step of the GA biosynthetic pathway are affected. The later stage of the GA biosynthesis pathway branches downstream of GA_{12}. The non-13-hydroxylation branch and the early 13-hydroxylation branch are parallel portions of the pathway that produce GA_4 and GA_1, respectively (Figure 1). We measured the GA levels of both branches in WT plant, *CmGA7ox* AS line, and *CmGA7ox* S line that show a high transcript levels. The amount of GA_{12}-aldehyde of 7-oxidase sense over-expression line in *Arabidopsis* plants were increased compared to wild type plant and transgenic antisense line (Figure 21a). The level of GA_{12} in WT plant was near in

CmGA7ox AS line. The GA_{12} level was dramatically increased in the 7-oxidase sense over-expressing line compared with those of wild type plant or transgenic antisense line. The non-13-hydroxylated metabolites GA_9 and GA_{25} were slightly elevated in *CmGA7ox* S line; however, the levels of GA_{15} and GA_{24} were similar to WT plant and antisense line. The levels of bioactive GA_4 and inactivated GA_{34} were slightly increased compared to control plants (Figure 21a).

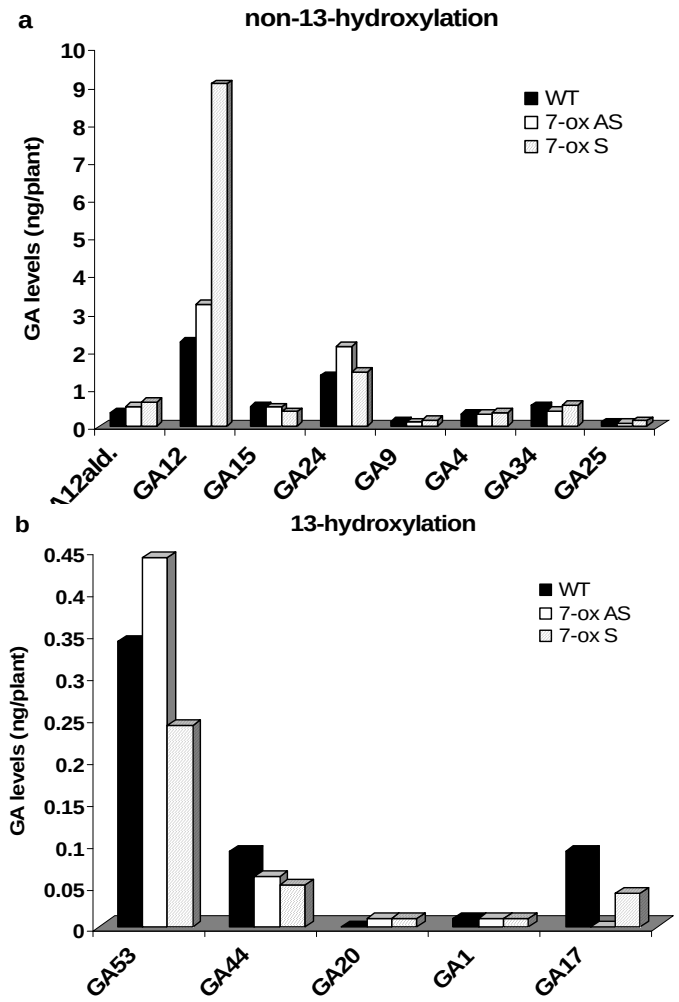


Figure 21: Endogenous GA levels in 7-week-old wild type (WT) and transgenic *Arabidopsis* plants expressing sense (S) or antisense (AS) copies of pumpkin 7-oxidase (*CmGA7ox*). a: non-13-hydroxylation pathway. b: 13-hydroxylation pathway.

Overall metabolite levels were higher in the non-13-hydroxylated pathway than in the parallel 13-hydroxylation pathway. This may be expected because GA_4 is the primary

active in *Arabidopsis* plants (Talón et al., 1990). In the 13-hydroxylated branch, the intermediate GA_1 and GA_{20} were unaffected in the *CmGA7ox* S line relative to WT plant and antisense line. However, the amount of GA_{53} was significantly different between WT plant and transgenic antisense line (Figure 21b).

The pumpkin GA 20-oxidase1 (*CmGA20ox1*) is known to produce inactive tricarboxylic acid GAs of no known physiological function (Lange, 1998). As shown in Figure 22a for non-13-hydroxylated GAs, the level of endogenous GA_4 (a biologically active GA) and its precursor GA_{24} and GA_9 were reduced in dwarf *CmGA20ox1* S line compared to WT plant, whereas, inactive GA_{25} tricarboxylic acid was increased. Moreover, the level of endogenous GA_{34} in *CmGA20ox1* sense line was increased relative to wild type plant, and GA_{12}-aldehyde together with GA_{12}, which initiated the first stage in non-hydroxylated pathway, were reduced. The content of GA_{20} and GA_1 in the dwarf line over-expressors *CmGA20ox1* were unaffected compared to WT plant. In addition, the level of GA_{17} was extremely increased in dwarf over-expressors line relative to wild type plant (Figure 22b).

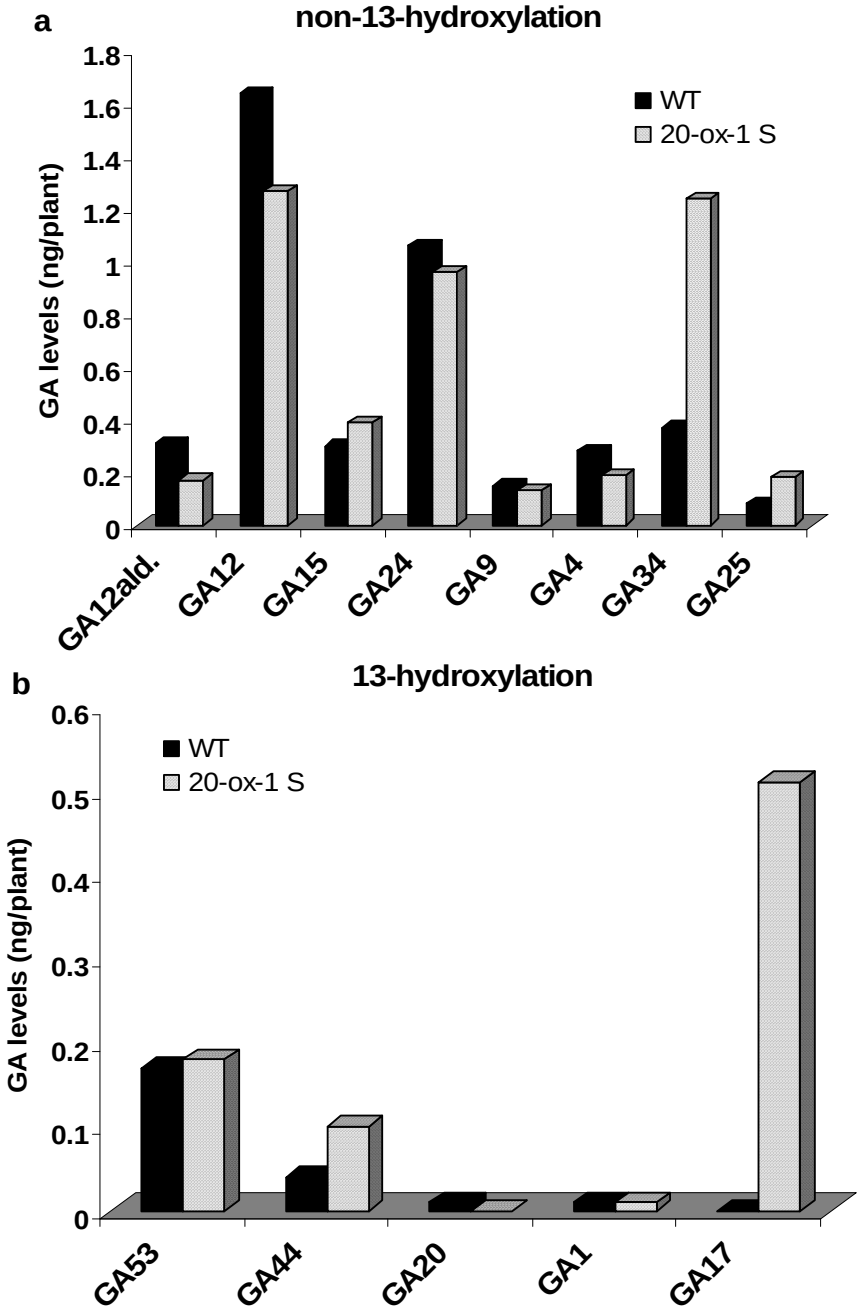


Figure 22: Endogenous GA levels in 7-week-old wild type (WT) and transgenic *Arabidopsis* plants expressing sense (S) copies of pumpkin 20-oxidase1 (*CmGA20ox1*). a: non-13-hydroxylation pathway. b: 13-hydroxylation pathway.

Arabidopsis slender plant over-expressing *CmGA3ox1* showed increased levels of endogenous GA_{12} as well as slightly increased levels of the precursors GA_{24} and GA_9 compared to AS line and WT plant. The level of endogenous GA_4 (a biologically active) and GA_{34} were increased relative to antisense line or WT plant (Figure 23a). .

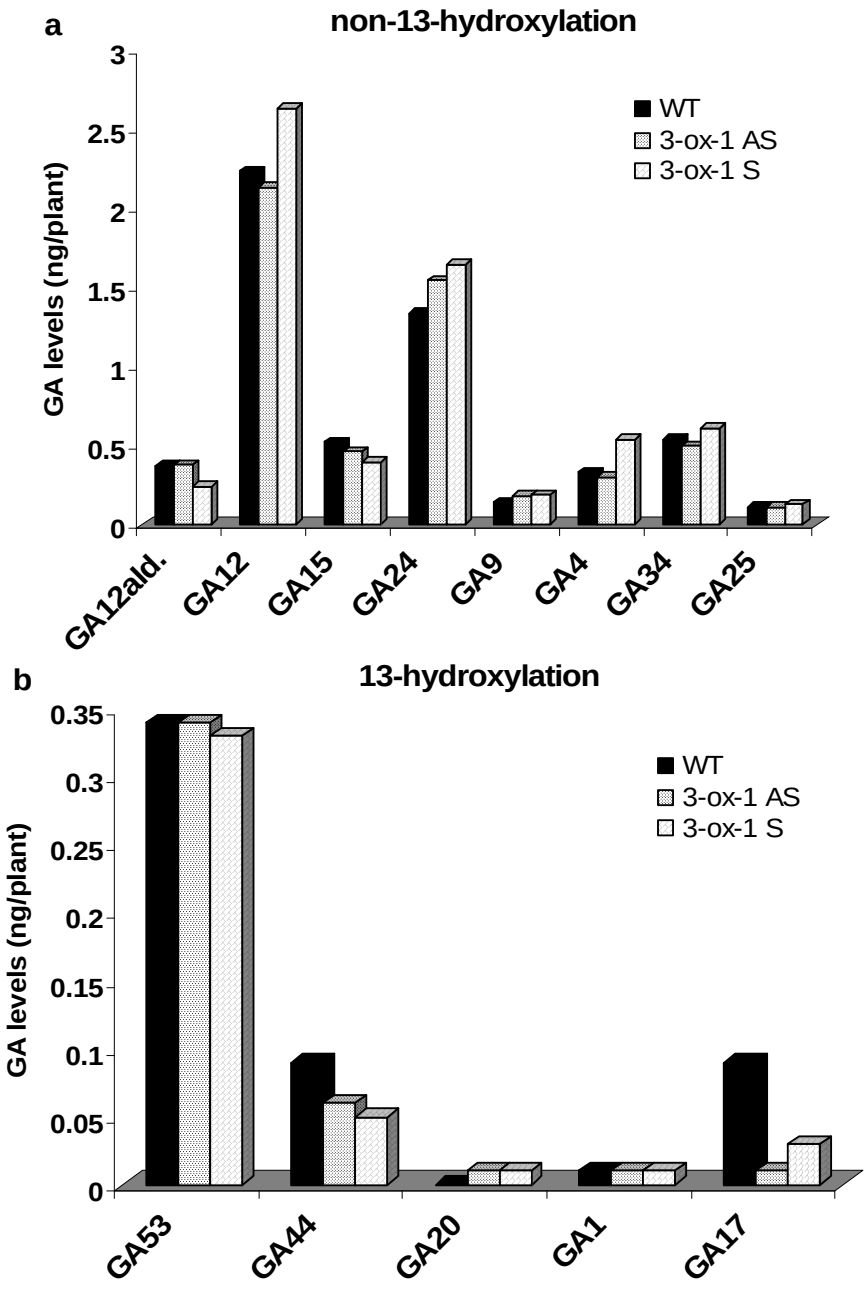


Figure 23: Endogenous GA levels in 7-week-old Wild type (WT) and transgenic *Arabidopsis* plants expressing sense (S) or antisense (AS) copies of pumpkin 3-oxidase1 (*CmGA3ox1*). a: non-13-hydroxylation pathway. b: 13-hydroxylation pathway.

In the 13-hydroxylated branch, the intermediate GA_{44}, and GA_{20} were not significantly different between transgenic sense line and control plants (Figure 23b). The level of GA_1 was unaffected in over-expressing *CmGA3ox1* S lines compared to control plants (Figure 23b).

We quantified GAs in shoot parts that might be affected by ectopic expression of the GA 2-oxidase1 (*CmGA2ox1*). Consistent with our expectations, the main bioactive GAs (GA_4) was substantially reduced in severe dwarf *CmGA2ox1* S line compared to control plants. GA_{34}, which is the inactive C-2 hydroxylated catabolites of GA_4, was higher in *CmGA2ox1* S line than in WT plant and AS line. Moreover, the levels of GA_{20} and GA_9 were decreased relative to WT plant and AS line. In addition, GA_{12}-aldehyde, GA_{12}, GA_{24}, and GA_9 were all present at low contents in *CmGA2ox1* S line (Figure 24a). GA_{53}, GA_{20}, and GA_1 were present in low amounts in sense line compared to WT plant. Whereas, there are no significant different in GA levels between antisense line and wild type plant (Figure 24b).

Table11: GA levels of early 3-oxidation pathway (ng/plant) in 7-week-old wild type (WT) and transgenic expressing sense (S) or antisense (AS) copies of *CmGA7ox*, *CmGA20ox1*, *CmGA3ox1*, and *CmGA2ox1*. *plants have been transferred to soil after 28 days in MS media containing 10^{-6}M GA_3. nd, no dilution of internal standard.

GAs	WT	WT*	7-ox AS	7-ox S	20-ox-1 S*	3-ox-1 AS	3-ox-1 S	2-ox-1 AS*	2-ox-1 S*
GA_{14}	0.01	nd	0.07	nd	nd	nd	nd	nd	nd
GA_{37}	0.03	nd	nd	0.01	0.01	0.01	0.01	nd	nd
GA_{36}	0.92	0.48	1.32	0.82	0.87	1.13	0.91	0.55	0.31

Table 11 shows the GA levels of the early 3-oxidation pathway (Figure 2). There was no significant difference between GA_{14} and GA_{37} in all transgenic lines compared to control plants. The level of GA_{36} was increased in *CmGA20ox1* S line relative to WT plant. In *CmGA2ox1* S line, the GA_{36} was decreased relative to control plants, whereas in *CmGA7ox* S line and *CmGA3ox1* S line GA_{36} was unaffected compared to control plants.

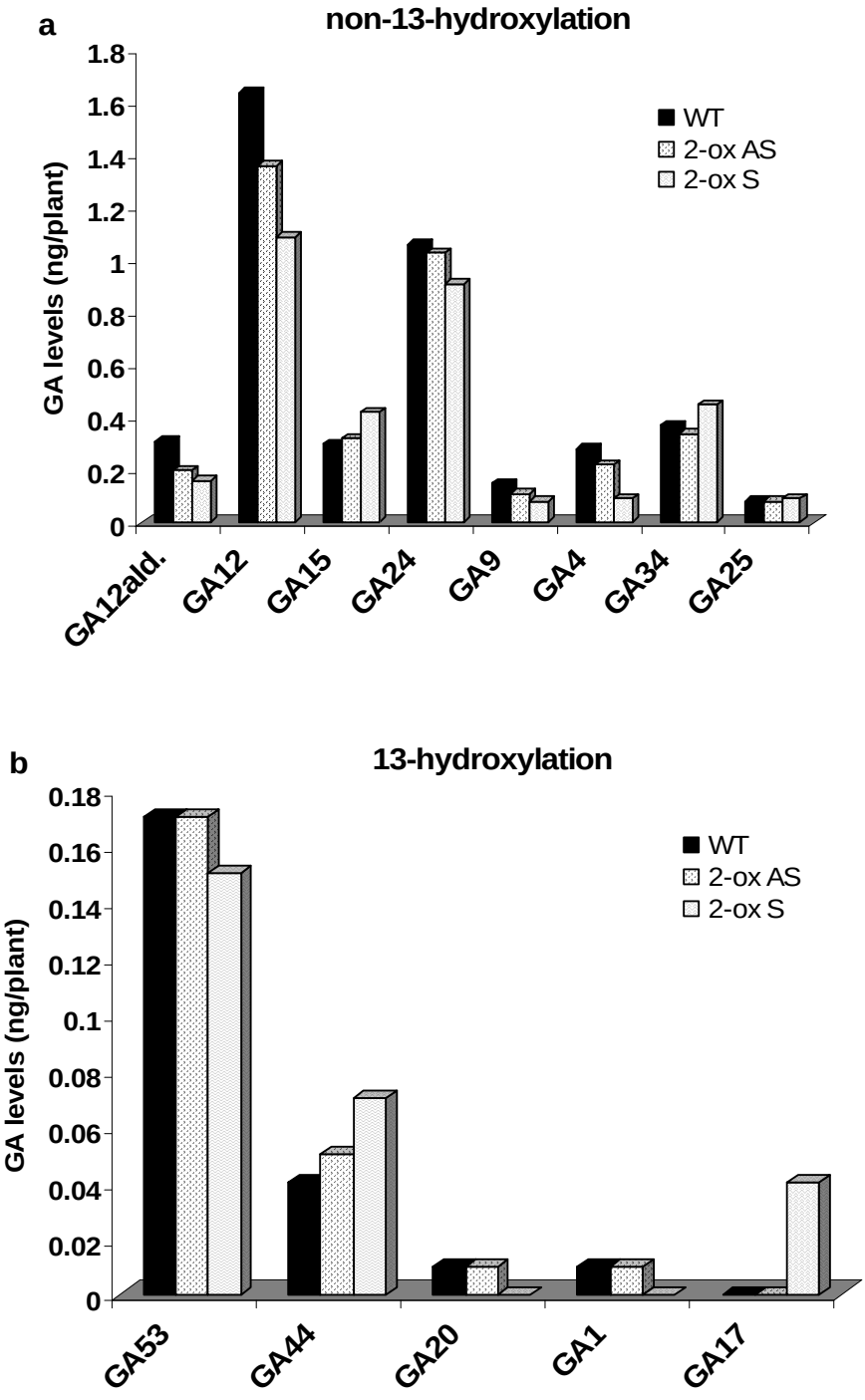


Figure 24: Endogenous GA levels in 7-week-old wild type (WT) and transgenic *Arabidopsis* plants expressing sense (S) or antisense (AS) copies of pumpkin 2-oxidase1 (*CmGA2ox1*). a: non-13-hydroxylation pathway. b: 13-hydroxylation pathway.

4. Discussion

Most genes encoding the enzymes of GA biosynthesis have been identified in *Arabidopsis* and other plant species. However, the function and the regulation of the GA pool is not entirely understood. Over-expression of the enzymes catalysing the final steps of the GA biosynthetic pathway may be a resource to help understanding the role of GAs in regulating plant development and elucidating the GA biosynthetic pathway. Pumpkin seeds contain GA-oxidases with singular catalytic properties resulting in GAs of unknown function for plant development.

In order to understand their potential to achieve changes in GA levels and their role for plant development, cDNA molecules of GA 7-oxidase (*CmGA7ox*), GA 20-oxidase1 (*CmGA20ox1*), GA 3-oxidase1 (*CmGA3ox1*) and GA 2-oxidase1 (*CmGA2ox1*) isolated from developing pumpkin seeds have been over-expressed in *Arabidopsis thaliana* ecotype Columbia under the control of a strong promoter cassette (E12-35S-Ω). The four pumpkin GA-oxidases that were studied in this thesis offer a suitable tool for manipulating GA biosynthesis, controlling plant development, and might therefore be useful in agriculture and horticulture.

Possible approaches for increasing bioactive GA levels in our transgenic plants include over-expression of *CmGA7ox* and *CmGA3ox1* (4.1., 4.2.; Figure 25). For reduction of GA levels by over-expression of *CmGA20ox1* and *CmGA2ox1* has been utilized (4.3., 4.4.; Figure 25).

4.1. Over-expression of *CmGA7ox*

Pumpkin contains GA 7-oxidase (CmGA7ox), a soluble dioxygenase, which oxidizes GA_{12}-aldehyde to GA_{12} and, as a side reaction, 3β-hydroxylates GA_{12} to GA_{14} (Lange, 1997; Frisse et al., 2003). The function of this soluble GA 7-oxidase is unclear and not found in other plant species. To study the influence of CmGA7ox on plant development and control of GA biosynthesis, cDNA molecules of *CmGA7ox* have been over-expressed in sense orientation under the control of strong promoter cassette in *Arabidopsis thaliana*. Wild type plants and antisense lines, obtained by transforming *Arabidopsis* with antisense copies of *CmGA7ox* were used as controls.

The homozygous transgenic lines were characterized in terms of height, internode length, number of siliques, final seed weight, and flowering time. The homozygous transgenic *Arabidopsis* seedlings expressing *CmGA7ox* germinated earlier and had a two- to three-fold increase in root length compared to control plants (Figure 10). Effects of gibberellins on root growth have been reported earlier (Tanimoto, 1994; Yaxley et al., 2001; Fu and Harberd, 2003). Recently, Oda et al. (2003) studied the effect of *XSP30* gene expression in the roots of cucumber (*Cucumis sativus*) by applying gibberellins to the shoot, suggesting that gibberellins are translocated to the roots or that gibberellins stimulate the production of a mediator in the shoot that causes a response in roots. In the case of the *Arabidopsis* GA-deficient mutant *ga1*-3, root growth can be regulated by the DELLA genes *RGA* and *GAI*, suggesting that GAs stimulate root growth by inducing the degradation of these DELLA proteins (Fu and Harberd, 2003; Fleet and Sun, 2005). Moreover, other hormones, for example, both auxin and ethylene that play a role in regulating the action of GAs in root growth, can affect GA-regulated root and hypocotyls growth by modifying the stability of RGA (Fu and Harberd, 2003; Achard et al., 2003).

At the later developmental stage, the homozygous transgenic plants had tall phenotypes, with longer internodes, early flowering, and more developed siliques compared to control plants (Figure 11, Table 10). RNA expression levels in different sense lines were determined by RT-PCR. The differences in the phenotypes can be attributed to the differences in the expression level of 7-oxidase sense lines with higher expression levels resulting in taller plants compared to control plants (Figure 11and 17).

Shoot parts of 7-week-old sense lines, antisense lines, and wild type plants were used for analysis of endogenous GAs. In the non-13-hydroxylated pathway, the *CmGA7ox* over-expressors resulted in an increase of GA_{12} content and only a slightly increase in the GA_4 content relative to wild type plants or transgenic antisense lines. GA_4 is the predominant active GA in *Arabidopsis* (Talón et al., 1990). Moreover, there are no differences in the endogenous GA levels of the 3β-hydroxylation pathway between sense lines and control plants indicating that the 3β-hydroxylation side activity of CmGA7ox has no apparent effect on GA biosynthesis in the transgenic *Arabidopsis* lines. In addition, of the early 13-hydroxylated pathway, GA_1 and GA_{20} contents were

unaffected in *CmGA7ox* sense lines, but GA_{53} levels decreased relative to transgenic antisense lines or wild type plants (Figure 21).

The above-mentioned small variations in GA contents might be because the whole shoot system was investigated. It is possible that by using different types of tissues or other developmental stages for the analysis of GA content would give increased differences in GA content between *CmGA7ox* over-expressors and the control plants. Coles et al. (1999), for example, found that the over-expression of GA 20-oxidase encoding genes in *Arabidopsis* plants gave rise to elongated hypocotyls of seedlings, with increase in shoot growth, and early flowering with no significant differences in the levels of GA_4 and GA_1 between shoot tips of the transgenic lines and wild type plants. On the other hand, when they analysed the endogenous GAs in rosette leaves only, they reported a two- to three-fold increase in the level of GA_4. It might also be that GA levels in *Arabidopsis* are closely regulated, so marked alternations in plant growth and development can be observed, while there are only small variations in the endogenous GA levels. These apparently small differences in GA content may have very significant physiological effects, as found in pea. In pea, it has been obtained that a relatively minor increase of GA_1 levels in the ovary produced maximum fruit development whereas much higher doses of GA_1 have to be treatment exogenously produce a same effect (Rodrigo et al. 1997).

The over-expression of *ent*-copalyl pyrophosphate synthase (CPS) and *ent*-kaurene synthase (KS) in *Arabidopsis*, which catalyse the first steps in GA biosynthesis, did not produce any effect on plant morphology but the level of *ent*-kaurene increased as well as the level of GA_{12} (Fleet et al ., 2003). The authors demonstrated that CPS is limiting for *ent*-kaurene production and suggested that the conversion of *ent*-kaurenoic acid (KA) to GA_{12} by *ent*-kaurenoic acid oxidase (KAO) may be an important rate-limiting step for production of bioactive GAs. They suggest that over-expressing KAO in combination with CPS and/or GA 20-oxidase might result in plants with higher levels of bioactive GA compared to plant that have been obtained by over-expressing GA 20-oxidase (Fleet et al., 2003).

Until now, only over-expression of GA 20-oxidases produced GA-overproduction phenotypes. In *Arabidopsis*, GA 20-oxidase over-expression had longer hypocotyls and petioles, larger rosette leaves, accelerated flowering and bolting, and longer stem,

as well as increased levels of bioactive GAs (Huang et al., 1998; Coles et al., 1999). The same results were obtained in potato (*Solanum tuberosum*; Carrera et al., 2000). In tobacco, over-expression of a citrus GA 20-oxidase cDNA showed shoot elongation with high increase in the level of GA_4 (Vidal et al., 2001). In addition, over-expression of *Arabidopsis* GA 20-oxidase showed that GAs not only affect plant elongation but also seems to effect biomass accumulation and lignin formation in transgenic tobacco plants (Biemelt et al., 2004). In hybrid aspen, over-expression of *Arabidopsis* GA 20-oxidase resulted in increased of bioactive GA levels, and taller trees (Eriksson et al., 2000).

The data presented show that it is possible to manipulate the plant stature by over-expressing pumpkin GA 7-oxidase in *Arabidopsis*, which produced GA-overproduction phenotypes with elongated internode, shoot, and early flowering as well as increased root growth. These results demonstrate that over-expression of pumpkin GA 7-oxidase can be utilized to alter GA levels and regulate growth and development in transgenic plants.

4.2. Over-expression of *CmGA3ox1*

It is well known that GA 3-oxidases convert inactive GA precursors to biological active GAs and it is important to control plant development in the life cycle of the plant (Lester et al., 1997; Williams et al., 1998; Itoh et al., 1999; Yamaguchi et al., 2001). In general, GA 3-oxidases catalyse reaction at the C-3β position of C_{19}-GAs to form biological active plant hormones (e.g. GA_4 or GA_1) (Figure 2). Further hydroxylation at C-2β position, catalysed by a GA 2-oxidase, leads to inactive products GA_{34} and GA_8, respectively. In pumpkin endosperm, a bi-functional GA 3-oxidase1 (CmGA3ox1) catalyses both steps, 3-oxidation and 2-oxidation, and prefers C_{20}-GAs to C_{19}-GAs as the substrate (Lange et al., 1997b). In order to understand the function of this seed specific GA 3-oxidase1, *CmGA3ox1* has been over-expressed under the control of a strong promoter cassette in *Arabidopsis*.

Over-expression of the seed specific *CmGA3ox1* leads to dramatic changes in plant growth and development (Figure 12, Table 10). *Arabidopsis* seedlings (14-day-old) over-expressing *CmGA3ox1* had increased leaf growth and contained thicker roots

with more lateral roots as compared to wild type plants (Figure 10). Over-expression of *CmGA3ox1* in *Arabidopsis* (7-week-old) resulted in phenotypes similar to the transgenic plants over-expressing *CmGA7ox*, a slender phenotype that flower earlier relative to wild type plants or transgenic antisense lines (Figure 12, Table 10). *CmGA3ox1* over-expressing sense lines showed early full maturity and even displayed an increase in the seed set relative to control plants. The difference in the slender phenotypes was due to the different *CmGA3ox1* expression levels (Figure 19). In addition, the internode length, the number of siliques, and the total seed weight per plant increased (Table 10). Furthermore, *CmGA3ox1* S17.7 line developed more siliques than the control plants. Interestingly, the number of siliques and the seed weight recorded per plant were even higher in *CmGA3ox1* in comparison with *CmGA7ox* over-expressors (Figure 15, Table 10). According to this observation, it development of the fruits in both type of over-expressors may proceed differently.

The analysis of endogenous GA levels in transgenic *CmGA3ox1* plants showed a two-fold increase in GA_4 content compared to control plants, as well as a slight increase in the inactive product GA_{34} (Figure 23). The high levels of GA_4 and the correlation between GA_4 and final shoot length suggest that GA_4 plays an important role in growth and development of the transgenic *Arabidopsis* plants. Furthermore, the GA_4-producing activity of CmGA3ox1 was higher than its GA_1-producing activity, suggesting that the CmGA3ox1 may stronger contribute to the non-13-hydroxylation pathway than to the early 13-hydroxylation pathway. The role of GA_4 as the predominant bioactive GA can be attributed to its higher concentrations as compared to GA_1 in *Arabidopsis* (Xu et al., 1997; Cowling et al., 1998).

On the other hand, a small increase in GA_4 levels was observed in *CmGA7ox* over-expressors, suggesting that the differences in plant development can be explained by the local modulation of GA levels in both *CmGA7ox* and *CmGA3ox1* over-expressors. The expression on rice genes involved in GA-biosynthesis and signaling, gives the indication that GAs are produced at the site where they act (reviewed by Sponsel and Hedden, 2004). Also on tobacco, *Nty* gene encoding 3β-hydroxylase is expressed at the site of GA action during stem elongation and flower organ development (Itoh et al., 1999).

The GA_{12}, GA_{24}, GA_9, and GA_{25} levels were elevated in *CmGA3ox1* over-expression lines compared to antisense lines and wild type plants (Figure 23). However, GA_{14}, GA_{37} and GA_{36} (early 3-oxidation pathway) were unchanged in comparison to antisense lines and wild type plants (Table 11). Some of endogenous GAs from the non-13-hydroxylation pathway increased while those of the early 3-oxidation pathway and early 13-hydroxylation pathway remained unaffected, suggesting that *Arabidopsis* plants over-expressing *CmGA3ox1* metabolises GAs mainly through the non-13-hydroxylation pathway, thus preventing them from being available for the 13-hydroxylated pathway.

Phillips (2004) reported that the over-expression of GA 3-oxidase in *Arabidopsis* had no effect on the development of transgenic plants. Moreover, Isaelsson et al. (2004) found that over-expression of a GA 3-oxidase from *Arabidopsis* in hybrid aspen resulted in increased 3β-hydroxylation activity but showed no major changes in the morphology of the plant and also found only small changes in GA_1 and GA_4 levels. The authors suggested that the limiting step in formation of GA_1 and GA_4 is 20-oxidation rather than 3-oxidation and that expression of GA 3-oxidase alone cannot increase the flux towards bioactive GAs.

The results presented here imply that GA 3-oxidase1 as well as GA 7-oxidase catalyses rate-limiting steps of the GA biosynthetic pathway in *Arabidopsis*. However, further investigations on hormone cross talk will allow us to understand how GAs and other hormone interact to control plant development of *CmGA7ox* and *CmGA3ox1* over-expressors.

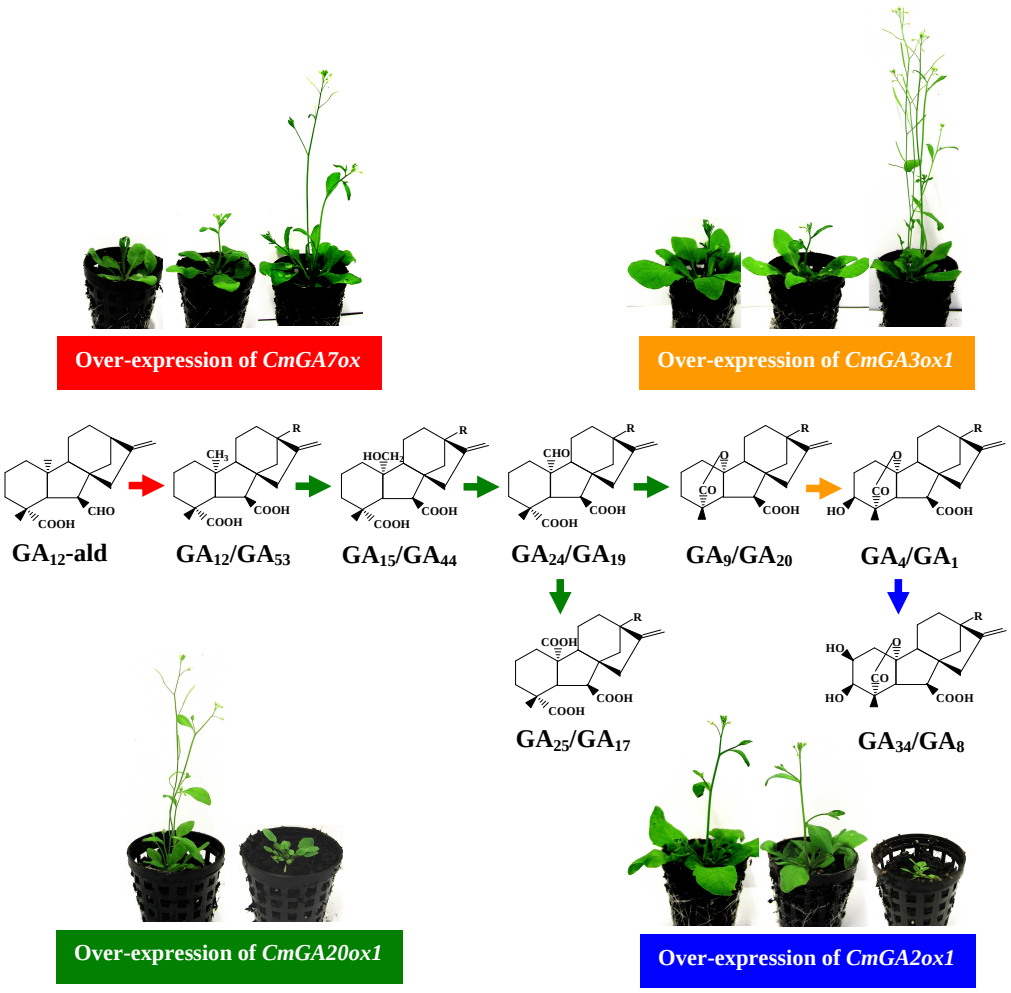


Figure 25: Strategies for manipulating plant development by increasing bioactive GA levels in transgenic plants, (over-expression of *CmGA7ox* and *CmGA3ox1*) or by reduction of GA levels (over-expressing of *CmGA20ox1* and *CmGA2ox1*).

4.3. Over-expression of *CmGA20ox1*

Mutants deficient in GA-biosynthetic enzymes have been shown to cause dwarfism in a variety of plant species (Martin et al., 1997; Hedden and Proebsting, 1999). In GA biosynthesis, GA 20-oxidase is regulated by both developmental and environmental stimuli (Phillips et al., 1995; Xu et al., 1995; Garcia-Martinez et al., 1997).

The normal activity of GA 20-oxidase is to carry out the sequential oxidation of GA_{12} and GA_{53} to GA_9 and GA_{20}, respectively (Figure 2). However, a seed specific GA 20-oxidase1 (CmGA20ox1) isolated from pumpkin seeds, is functionally different from other GA 20-oxidases. It converted C_{20}-GAs to C-20 carboxylic acid GA_{25} and GA_{17}.

GA_{25} and GA_{17} have no known physiological function, rather than C_{19}-GAs (GA_9 and GA_{20}) (Lange, 1994, 1998; Lange et al., 1994b; Frisse et al., 2003). Theoretically, over-expression of this enzyme in tissues involved in GA biosynthesis should change the pathways leading to inactive products and by that reduce the levels of bioactive GAs resulting in dwarf plants. Several groups using different plant species tested this strategy and produced divert results.

In *Arabidopsis* ecotype Wassilewskija, over-expression of *CmGA20ox1* resulted in reduction of GA_4 levels and unaffected GA_1 levels but only a slight reduction in the height of the transgenic plants (Xu et al., 1999). The authors argued that the reduction of stem elongation required a large reduction in the content of GA_4. Curtis et al. (2000) were successful in producing semi-dwarf phenotypes of *Solanum dulcamara* by over-expression of *CmGA20ox1*. In their transgenic lines GA_1 levels were reduced but GA_4 levels were unaffected and this indicated that in this plant the 13-hydroxylation pathway is preferred. It was demonstrated that a feed back control mechanism in GA biosynthesis, resulting in up-regulation of endogenous GA 20-oxidase gene (*Arabidopsis* and *Solanum*) and GA 3-oxidase gene was accountable for the non-success in reducing plant height. However, in lettuce, Niki et al. (2001) obtained dwarf plants with high reduction of bioactive GA_1 and GA_4 and large accumulation of GA_{17} and GA_{25}, which are inactive products by over-expression of pumpkin GA 20-oxidase1.

We over-expressed sense copies of pumpkin GA 20-oxidase1 (*CmGA20ox1*) in *Arabidopsis thaliana* ecotype Columbia under the control of a strong constitutive promoter cassette (E12-35S-Ω), similar to one used to express *CmGA20ox1* in lettuce (Niki et al., 2001). The phenotypes of homozygous transgenic lines over-expressing *CmGA20ox1* were dwarfed compared to the wild type plants. Morphological changes showed a reduction of rosette leaf size and shoot height (Figure 13). Furthermore, we observed that flowering was delayed and silique production was reduced compared to wild type plants (Table 10). Moreover, seed dormancy was observed in both heterozygous and homozygous lines, and these seeds, in contrast to wild type plants, germinated only in the presence of GA_3. This behaviour is similar to GA-deficient mutants of *Arabidopsis* and tomato, where the application of GA is required for a full germination response (Koornneef and Van der Veen, 1980; Groot and Karssen, 1987;

Karssen et al., 1989, Derkx and Karssen 1994). In contrast, in the case of *Solanum dulcamara*, the semi-dwarfed plants over-expressing the pumpkin GA 20-oxidase1 flowered earlier and produced more fruit and seeds (Curtis et al., 2000).

To determine the expression levels of the transgene in *Arabidopsis*, mRNA levels were quantified from rosette leaves by RT-PCR. The results obtained showed, that the *CmGA20ox1* S17.2 line had the highest transcript level (10 µg/g total RNA) (Figure 18). This line expressed a dwarf phenotype (Figure 13) and the dwarfed plants had reduced levels of the biologically active GA_4 (Figure 22). In the other lines, transcript levels were not found for *CmGA20ox1* S10.8 and S2.2 lines. The phenotype of *CmGA20ox1* S10.8 line was nearly unchanged compared to wild type plants. However, *CmGA20ox1* S2.2 line had dwarfed phenotype.

It is well known that GA 20-oxidase can contribute to the two branches of GA biosynthetic pathway; the non-13-hydroxylation and the early 13-hydroxylation pathway, which convert GA_{12} to GA_9 and GA_{53} to GA_{20}, respectively (Figure 2). In pumpkin, GA 20-oxidase converts more effectively the substrates of the non-13-hydroxylated pathway than of the early 13-hydroxylated pathway (Lange et al., 1994b). In our experiments, over-expression of *CmGA20ox1* in *Arabidopsis* resulted in reducing most of the endogenous GAs of the non-13-hydroxylated pathway including GA_4, by increasing the level of the tri-carboxylic GA_{25}. The concentrations of GAs of the early-13-hydroxylated pathway were similar or unchanged relative to wild type plants, except for GA_{17} where a high accumulation was observed, being the amount of GA_{17} higher than the amount of GA_{25} (Figure 22). The transgenic *Arabidopsis* plants over-expressing *CmGA20ox1* had increased levels of the tri-carboxylic GA_{25} and GA_{17}, and reduced bioactive GA_4 levels resulting in dwarfed phenotypes consistent with those obtained when the same gene was over-expressed in *Arabidopsis* (Xu et al., 1999). Furthermore, the elevated levels of GA_{34} indicate the presence of an increased 2-oxidation activity operating in the transgenic plants.

Expressing pumpkin GA 20-oxidase1, which convert the biosynthetic pathway to inactive products, is one possible strategy for reducing GA content and plant height. We succeeded in producing dwarf *Arabidopsis* plants using this strategy that was also used successfully in lettuce (Niki et al., 2001) and partially in *Solanum ducamara* (Curtis et al., 2000). Other approaches have been made to reduce the levels of

bioactive GAs including antisense expression of GA 20-oxidase in *Arabidopsis* and potato (Coles et al., 1999; Carrera et al., 2000). Over-expression of KNOTTED-1 class of homeobox transcription factors may reduce the expression level of *GA20ox* genes. For instance, over-expression of *NTH15*, a tobacco *KNOX* gene, in transgenic tobacco decreases expression of endogenous GA 20-oxidase genes, results in reduced GA levels and abnormal leaf and flower morphology (Sakamoto et al., 2001). Antisense suppression of GA 3-oxidase genes was also effective in reducing GA levels in rice plants (Itoh et al., 2002). The transgenic rice plants by over-expressing antisense copies of *OsGA3ox2* had reduced expression of the target gene and resulted in a semi-dwarf phenotype. Our results showed that over-expressing *CmGA20ox1* under the control of strong promoter cassette is a useful strategy, which can be extended to other plant species, for reduction of GA content and control of plant development.

4.4. Over-expression of *CmGA2ox1*

The balance between synthesis and catabolism controls the pool of bioactive GAs. GA 2-oxidase is a catabolic enzyme that catalyses the conversion of bioactive GAs into inactive GAs by 2β-hydroxylation (Ross et al., 1995). The application of GA_3 was reported to stimulate the expression of GA 2-oxidase genes in *Arabidopsis*, implying that the expression of these genes may be regulated through feed-forward mechanisms to maintain endogenous levels of bioactive GAs (Thomas et al., 1999). The authors found that two of three studied *Arabidopsis* 2-oxidase were most abundant in the inflorescence and developing siliques. This expression pattern was consistent with the role of GA 2-oxidases in reducing GA levels in seeds to promote dormancy. Martin et al. (1999) provided another evidence for this role upon studying the *SLENDER* gene of pea, which also encodes GA 2-oxidase. They observed hyper-elongation of slender mutant phenotype in seedlings, resulting in high levels of GA precursors in seeds, which are converted to active GAs upon germination.

Over-expression of genes encoding GA 2-oxidase catabolic enzyme offer another possible approach to decrease GA levels and reduce plant height (Thomas et al., 1999; Schomburg et al., 2003; Sponsel and Hedden, 2004). Genetic manipulation of GA 2-oxidase encoding genes has been carried out in different plant species. In rice, over-expression of GA 2-oxidase resulted in inhibition of stem growth, small, dark green

leaves, and decayed development of reproductive organs (Sakamoto et al., 2001). Over-expression of poplar GA2ox (*PtaGA2ox1*) caused a dwarf transgenic hybrid poplar (Busov et al., 2003). Moreover, over-expression of *Arabidopsis* GA 2-oxidase in tobacco produced dwarfed phenotypes and it was found that GA not only affects dwarf phenotype but also seems to affect biomass accumulation and lignin formation (Biemelt et al., 2004). Over-expression of two of the eight *Arabidopsis* GA 2-oxidases (*AtGA2ox7* and *AtGA2ox8*), that hydroxylate C_{20}- instead of C_{19}-GA precursors, showed decreased levels of active GAs and corresponding dwarf phenotypes in *Arabidopsis* (Schomburg et al., 2003).

In pumpkin, only one GA 2-oxidase1 gene (*CmGA2ox1*) is known and its recombinant protein converts C_{19}-GAs as a precursors. It efficiently inactivates both, bioactive GA_1 and GA_4 (Frisse et al., 2003). To study the impact of *CmGA2ox1* on altering GA biosynthesis and, by this, plant growth and development, we have over-expressed the pumpkin GA 2-oxidase1 in *Arabidopsis* plants. The morphological characteristics of *Arabidopsis* plants transformed with sense copies of *CmGA2ox1* were similar to the phenotype of the deficient mutants that contain defective GA biosynthetic genes. They share severely reduced stem elongation, decreased leaf size, and dark green colour (Sun and Kamiya, 1994; Helliwell et al., 1998; Yamaguchi et al., 1998; Schomburg et al., 2003).

The transgenic plants obtained had severe dwarfed phenotypes and delayed flowering. The leaf base was reduced in length resulting in leave clusters (Figure 14). The severe dwarf transgenic line (S12.9) had a dramatic decrease in the number of siliques, and seed weight per plant compared to wild type plants and antisense transgenic lines (Figure 15, Table 10). The phenotype of plants expressing antisense copies of *CmGA2ox1* did not change compared to wild type plants. The difference in the phenotypic severity was due to the different *CmGA2ox1* expression levels (Figure 20). In rice, over-expression of GA 2-oxidase (*OsGA2ox1*), using rice actin promoter, resulted in severe dwarf phenotypes and inhibited development of reproductive organs (Sakamoto et al., 2001). However, over-expression of the same gene under the control of shoot specific promoter *OsGA3ox2*, showed semi-dwarf phenotypes but the flowering and grain development were unaffected (Sakamoto et al., 2003).

The transgenic GA 2-oxidase1 (*CmGA2ox1*) lines examined exhibited a disturbed segregation ratio of T_2 and T_3 generation seeds compared to wild type plants and antisense transgenic lines. Exogenous application of GA_3, which is resistant to catabolism by GA 2-oxidase, rapidly restored germination to resistant lines, strongly suggesting that the dwarf line is a result of the deficiency of the bioactive GAs. The physiological role of GAs in *Arabidopsis* seed germination is also in agreement with previous results suggesting that the GAs are required for normal seed germination in the *gib1* tomato mutant (Groot et al., 1987) and in GA-deficient mutants of barley (Chandler and Robertson, 1999).

In previous studies, over-expression of runner bean *GA2ox* gene, *PcGA2ox1*, in transgenic wheat under the maize promoter produced a range of phenotypes including semi dwarf and severe dwarf plants (Phillips, 2004). Over-expression of the same gene in sugar beet, to increase resistance to vernalisation, showed reduction of leaf expansion and dwarf phenotype. However, the transgenic plants grown under inductive conditions bolted at a similar time than to controls, but the flowers were infertile (Phillips, 2004). Sakai et al. (2003) over-expressed the novel rice gibberellin 2-oxidase gene (*OsGA2ox3*) and showed an extremely dwarfed phenotype in rice.

In our results, analysis of GA content in severely dwarf GA 2-oxidase1 (*CmGA2ox1*) *Arabidopsis* plants showed decreased levels of the bioactive GA_4 compared to control plants, and an increase of inactive GA_{34}. The levels of GA_{12}-aldehyde and GA_{12} were decreased in comparison to antisense lines and wild type plants. GA contents of the intermediated precursors (GA_{15}, GA_{24}, GA_9, and GA_{25}) were decreased of the non-13-hydroxylated GAs pathway (Figure 24). The results presented show that it is possible to manipulate the plant stature by over-expression of *CmGA2ox1*, which led to a reduction of GA_4 content, an increase in inactive products GA_{34} and to severely dwarfed phenotypes in *Arabidopsis*. GA 2-oxidase may provide a strategy for the development of dwarf varieties of plant species.

In conclusion, our study shows that over-expression of the *CmGA7ox* and *CmGA3ox1* results in increase GA levels with extremely elongated phenotypes in *Arabidopsis*, showing that both enzymes catalyse rate-limiting steps in the GA biosynthesis of *Arabidopsis*. In contrast, over-expression of *CmGA20ox1* and *CmGA2ox1* in *Arabidopsis* results in dwarf plants with decreased GA levels. The four pumpkin GA-

oxidases exploited in this study, may therefore offer useful tools for controlling plant stature in other agricultural and horticultural species.

5. Summary

Manipulation of plant stature has been a major tool in agriculture, horticulture, and forest culture. It previously has involved plant breeding and the use of plant growth regulators produced by the chemical industry. These are exogenously applied to promote or retard elongation, through chemical alteration of GA biosynthesis. Biotechnological manipulation of GA levels provides an alternative approach and can be achieved through various means, including up- or down-regulating genes encoding enzymes involved in GA biosynthesis and catabolism.

To elucidate the effect of GA biosynthetic enzymes on plant growth, development, and on GA levels, we expressed sense copies of cDNA molecules encoding GA 7-oxidase (*CmGA7ox*), GA 3-oxidase1 (*CmGA3ox1*), GA 20-oxidase1 (*CmGA20ox1*) and GA 2-oxidase1 (*CmGA2ox1*) in *Arabidopsis thaliana* ecotype Columbia under the control of a strong promoter cassette (E12-35S-Ω). Wild type plants and antisense lines, obtained by transforming *Arabidopsis* with antisense copies of respective GA-oxidases were used as controls. The constructed binary vectors were transformed with *Agrobacterium tumefaciens* and introduced into wild type plants using floral dip transformation. T_2 seeds were screened for 3:1 (resistant: sensitive) kanamycin resistance. Transgenic lines were re-segregated at T_3 generation to identify homozygous lines.

The results presented show that it is possible to manipulate the plant stature by over-expression of *CmGA7ox* and *CmGA3ox1* genes that lead to an increase of GA_4 content and to an extremely elongated phenotype in *Arabidopsis thaliana*. Phenotypic characteristics conferred by the over-expression of GA 7-oxidase can be visualized at early stages of seedling growth, by increased of leaf growth and root elongation. At the later developmental stage, the transgenic lines had taller phenotypes with longer internodes that flower earlier, and develop more siliques relative to control plants. Our RT-PCR analysis showed high transcript levels at the three different lines of GA 7-oxidase over-expressors. The differences in the phenotype observed correlated to the different expression levels of the 7-oxidase gene. Over-expression of GA 7-oxidase in transgenic *Arabidopsis* plants resulted in an increase in GA_{12} levels and a slightly increase of GA_4 levels. In addition, we successfully isolated three transgenic lines of

GA 3-oxidase1; one of the homozygous lines (S17.7) had a very tall phenotype with higher levels of GA_4 compared to control plants. Furthermore, these transgenic plants expressed high transcript levels (1000µg/g). Generally, the transgenic plants over-expressing GA 3-oxidase1 sense lines developed more siliques and mature earlier than the GA 7-oxidase sense lines.

Over-expression of the pumpkin GA 20-oxidase1 resulted in altered leaf morphology, dwarfism and decreased silique production and seed set. Leaves were small and dark green. The petioles were reduced in length resulting in leaves closer together. Over-expression lines exhibited dwarfism as a result of reduced internode length. The most severe dwarf plants correlated with the highest levels of pumpkin GA 20-oxidase1 transcript. Transgenic lines share many aspects of the phenotypes obtained with GA-deficient mutants including dwarfism, reduced internode elongation, and promoted seed dormancy. Exogenous application of GA_3 rescued germination of homozygous and heterozygous seeds, indicating that over-expression lines were responsive to GA. The levels of bioactive GA were reduced in over-expression lines with inactive GA products (GA_{17} and GA_{25}) accumulating. The use of pumpkin GA 20-oxidase1 to divert the GA biosynthetic pathway to inactive products is an attractive strategy for reducing GA content and plant stature.

In another manner, over-expressing genes encoding GA-catabolizing enzymes, such as pumpkin GA 2-oxidase1 results in dwarfed plants. Transgenic *Arabidopsis* expressing pumpkin GA 2-oxidase1 showed a range of phenotypes. These severe dwarfs developed darker green, wider, and shorter leaves. The difference in the phenotypic severity was due to the difference in the GA 2-oxidase1 expression levels, which were estimated by RT-PCR. Finally, in GA 2-oxidase1 over-expressing plants GA_{34} levels (catabolic product of GA_4) increased, whereas the GA_4 levels decreased. In general, the phenotype of plant expressing antisense copies of the respective pumpkin GA-oxidases studied here did not change compared to the wild type plants. Our results demonstrate that expression of pumpkin GA-oxidases can be used to alter GA levels and by this regulate growth and development in transgenic plants.

6. Literature Cited

Aach H, Bode H, Robinson DG, Graebe JE (1997) Ent-kaurene synthase is located in proplastides of meristematic shoot tissues. Planta 202: 211-219

Aach H, Böse G, Graebe JE (1995) Ent-kaurene biosynthesis in a cell-free system from wheat (Triticum aestivum L.) seedlings and the localization of ent kaurene synthetase in plastids of three species. Planta 197: 333-342

Achard P, Vriezen WH, Van Der Straeten D Harberd NP (2003) Ethylene regulates *Arabidopsis* development via the modulation of DELLA protein growth repressor function. Plant Cell 15: 2816-2825

Albone KS, Gaskin P, MacMillan J, Phinney BO, Willis CL (1990) Biosynthetic origin of gibberellin A_3 and gibberellin A_7 in cell-free preparations from seeds of *Marah macrocarpus* and *Mallus domestica*. Plant Physiol 94: 132-142

Barlow JN, Baldwin JE, Clifton IJ, Gibson E, Hensgens CMH, Hajdu J, Hara T, Hassan A, John P, Lloyd MD, Roach PL, Prescott A, Robinson JK, Zhang ZH, Schafield CJ (1997) Studies on non-haem ferrous-dependent oxygenases and oxidases. Biochem Soc Trans 25: 86-90

Biemelt S, Tschiersch H, Sonnewald U (2004) Impact of altered gibberellin metabolism on biomass accumulation lignin biosynthesis, and photosynthesis in transgenic tobacco plants. Plant Physiol 135: 254-265

Birnboim HC, Doly J (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl Acids Res 6: 1513-1523

Boliver F, Backman K (1979) Plasmids of *Escherichia coli* as Cloning Vectors. Methods Enzymol 68: 245-267

Boyer HW, Roulland-Dussoix D (1969) A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol 41: 459-472

Brian PW, Hemming HG (1955) The effect of gibberellic acid on shoot growth and development. Physiol Plant 8: 669-681

Bullock WO, Frenandez JM, Stuart JM (1987) XL 1-Blue: A high efficiency plasmid transforming *recA Escherichia coli* strain with beta-glactosidase selection. Biotechniques 5: 375-379

Busov VB, Meilan R, Pearce DW, Ma C, Rood SB, Strauss SH (2003) Activation tagging of a dominant gibberellin catabolism gene (GA 2-oxidase) from Poplar that regulates tree stature. Plant Physiol 132: 1283-1291

Carrera E, Bou J, Garcia-Martinez JL, Prat S (2000) Changes in GA 20-oxidase gene expression strongly affect stem length, tuber induction and tuber yield of potato plants. Plant J 22: 247-256

Chandler PM, Robertson M (1999) Gibberellin dose-response curves and the characterization of dwarf mutants of barley. Plant Physiol 120: 623-632

Chiang HH, Hwang I, Goodman HM (1995) Isolation of the *Arabidopsis GA4* locus. Plant Cell 7: 195-201

Christadoulou AJ, Weaver RJ, Pool RM (1968) Relation of gibberellin treatment to fruit-set, berry development and cluster compactness in *Vitis vinifera* grapes. Proc Am Soc Hort Sci 92: 301-310

Clough SJ, Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J 16: 735-743

Coles JP, Phillips AL, Croker SJ, Garcia-Lepe R, Lewis MJ, Hedden P (1999) Modification of gibberellin production and plant development in *Arabidopsis* by sense and antisense expression of gibberellin 20-oxidase. Plant J 17: 547-556

Cowling RJ, Kamiya Y, Seto H, Harberd NP (1998) Gibberellin dose-response regulation of GA_4 gene transcript levels in *Arabidopsis*. Plant Physiol 117: 1195-1203

Creelman RA, Mullet JE (1997) Oligosaccharines, brassinolids and jasmonates: Non-traditional regulators of plant growth, development and gene expression. Plant Cell. 1997; 9 (7): 1211-1223

Crozier A (1983) The biochemistry and physiology of Gibberellins, Vol 2. Praeger, New York

Curtis IS, Ward DA, Thomas SG, Phillips AL, Davey MR, Power JB, Lowe KC, Croker SJ, Lewis MJ, Magness SL, Hedden P (2000) Induction of dwarfism in transgenic *Solanum dulcamara* by over-expression of gibberellin 20-oxidase cDNA from pumpkin. Plant J 23: 329-338

Curtis PJ, Cross BE (1954) Gibberellin acid. A new metabolite from the culture filtrates of *Gibberella fujikuroi*. Chem Ind 1066

Davis RW, Botstein D, Roth JR (1980) Advanced bacterial genetics, a manual for genetic engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, USA

De Carolis E, De Luca V (1994) 2-oxoglutarate-dependent dioxygenases and related enzymes: Biochemical characterisation. Phytochemistry 36: 1093-1107

Derkx MPM, Karssen CM (1994) Are seasonal dormancy patterns in *Arabidopsis thaliana* regulated by changes in seed sensitivity to light, nitrate and gibberellin? Ann Bot 73: 129-136

Eriksson ME, Israelsson M, Olsson O, Mortiz T (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. Nat Biotechnol 18: 784-788

Eriksson ME, Moritz T (2002) Day length and spatial expression of a gibberellin 20-oxidase isolated from hybrid aspen (*Populus tremula* L. x *P. tremuloides Michx*.) Planta 214: 920-930

Fleet CM, Sun T-p (2005) A DELLAcate balance: the role of gibberellin in plant morphogenesis. Curr Opin Plant Biol 8: 1220-1227

Fleet CM, Yamaguchi S, Hanada A, Kawaide H, David CJ, Kamiya Y, Sun TP (2003) Overexpression of *At*CPS and *At*KS in *Arabidopsis* confers increased ent-kaurene production but no increase in bioactive gibberellins. Plant Physiol 132: 830-839

Freeman WM, Walker SJ, Vrana KE (1999) Quantitative RT-PCR: Pitfalls and potential. Bio Techniques 26: 112-125

Frisse A (1999) Untersuchungen zur expression von gibberellin dioxygenase genen in kürbiskeimlingen. Doktorgrades Georg-August- Universität, Göttingen

Frisse A, Pimenta MJ, Lange T (2003) Expression studies of gibberellin oxidases in developing pumpkin seeds. Plant Physiol 131: 1220-1227

Fu X, Harberd P (2003) Auxin promotes *Arabidopsis* root growth by modulating gibberellin response. Nature 421: 740-743

Garcia-Martinez JL, Lopez-Diaz I, Sanchez-Beltran MJ, Phillips AL, Ward DA, Gaskin P, Hedden P (1997) Isolation and transcript analysis of gibberellin 20-oxidase genes in pea and bean in relation to fruit development. Plant Mol Biol 33: 1073-1084

Gough J, Murray N (1983) Sequence diversity among related genes for recognition of specific targets in DNA molecules. J Mol Biol 166: 1-19

Graebe JE (1987) Gibberellin biosynthesis and control. Annu Rev Plant Physiol 38: 419-465

Graebe JE, Ropers HJ (1978) Gibberellins. In: Letham DS, Goodwin PB, Higgins TJV (eds) Phytohormones and related compounds - a comprehensive treatise. Vol 1. Elsevier Amsterdam, pp 107-188

Groot SPC, Karssen CM (1987) Gibberellins regulate seed germination in tomato by endosperm weakening: a study with gibberellin-deficient mutants. Planta 171: 525-531

Hanna Z, Fregau C, Prefontain G, Broussau R (1984) Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 166: 557-580

Hedden P (1999) Regulation of gibberellin biosynthesis. In Biochemistry and Molecular Biology of Plant Hormones (Hooykaas PPJ, Hall MA, Libbenga KR eds). New York: Elsevier Science, pp 161-188

Hedden P, Coles JP, Phillips AL, Thomas SG, Ward DA, Curtis IS, Power JB, Lowe KC, Davey MR (1998) Modification of plant morphology by genetic manipulation of gibberellin biosynthesis. In: Cockshull KE, Gray D, Seymour GB, Thomas B, eds. Genetic and environmental manipulation of horticultural crops, Wallingford: CAB International, 205-217

Hedden P, Hoad G (1994) Groth regulators and crop productivity. In Mechanisms of plant groth and improved productivity: Modern Approaches (Basra AS, ed). New York: Marcel Dekker, pp. 173-198

Hedden P, Kamiya Y (1997) Gibberellin biosynthesis: enzymes, genes and their regulation. Annu Rev Plant Physiol Plant Mol Biol 48: 431-460

Hedden P, Phillips AL (2000a) Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci 5: 523-530

Hedden P, Phillips AL (2000b) Manipulation of hormone biosynthetic genes in transgenic plants. Curr Opin Biotechnol 11: 130-137

Hedden P, Proebsting WM (1999) Genetic analysis of gibberellin biosynthesis. Plant Physiol 119: 365-370

Hedden P, Phillips AL, Rojas MC, Carrera E, Tudzynski B (2002) Gibberellin biosynthesis in plants and Fungi: A case of convergent evolution? J Plant Growth Regul 20: 319-331

Helliwell CA, Chandlar PM, Poole A, Dennis ES, Peacock WJ (2001a) The CYP884 cytochrome P450, ent-kaurenoic acid oxidase, catalyses three steps of the gibberellin biosynthesis pathway. Proc Natl Acad Sci USA 98: 2065-2070

Helliwell CA, Poole A, Peacock WJ, Dennis ES (1999) *Arabidopsis* ent kaurene oxidase catalyses three steps of gibberellin biosynthesis. Plant Physiol 119: 507-510

Helliwell CA, Sheldon CC, Olive MR, Walker AR, Zeevaart JAD, Peacock WJ, Dennis ES (1998) Cloning of the *Arabidopsis* ent-kaurene oxidase gene GA3. Pro Natl Acad Sci USA 95: 9019-9024

Helliwell CA, Sullivan JA, Mould RM, Gray JC, Peacock J, Dennis ES (2001b) A plastid envelope location of *Arabidopsis* ent-kaurene oxidase links the plastid and endoplasmic reticulum steps of the gibberellin biosynthesis pathway. Plant J 28: 201-208

Huang S, Raman AS, Ream JE, Fujiwara H, Cerny RE, Brown SM (1998) Overexpression of 20-oxidase confers a gibberellin-overproduction phenotype in *Arabidopsis*. Plant Physiol 118: 773-781

Israelsson M, Mellerowicz E, Chona M, Gullberg J, Moritz T (2004) Cloning and overproduction of gibberellin 3-oxidase in hybrid aspen trees. Effects on gibberellin homeostasis and development. Plant Physiol 135: 221-230

Itoh H, Ueguchi-Tanaka M, Kawaide H, Chen X, Kamiya Y, Matsuoka M (1999) The gene encoding tobacco gibberellin 3β-hydroxylase is expressed at the site of GA action during stem elongation and flower organ development. Plant J 20: 15-24

Itoh H, Ueguchi-Tanaka M, Sakamoto T, Kayano T, Tanaka H, Ashikari M, Matsuoka M (2002) Modification of rice plant height by suppressing the height-controlling gene, D18, in rice. Breeding Science 52: 215-218

Kamiya Y, Graebe JE (1983) The biosynthesis of all major pea gibberellins in a cell-free system from *Pisum sativum*. Phytochemistry 22: 681-689

Kang HG, Jun SH, Kim J, Kawaide H, Kamiya Y, An G (1999) Cloning and molecular analyses of gibberellin 20-oxidase gene expressed specifically in developing seeds of watermelon. Plant Physiol 121: 373-382

Kang HG, Jun SH, Kim J, Kawaide H, Kamiya Y, An G (2002) Cloning of gibberellin 3β-hydroxylase cDNA and analysis of endogenous gibberellins in the developing seeds in watermelon. Plant Cell Physiol 43: 152-158

Karssen CM, Zagorski S, Kepczynski J, Groot SPV (1989) Key role for endogenous gibberellins in the control of seed germination. Ann Bot 63: 71-80

Kende H, Zeevaart JAD (1997) The five "classical" plant hormones. Plant Cell 9: 1197-1210

Kobayashi M, Spray CR, Phinney BO, Gaskin P, MacMillan J (1996) Gibberellin metabolism in maize: The stepwise conversion A_{12}-aldehyde to gibberellin A_{20}. Plant Physiol 110: 413-418

Koornneef M, Van der Veen JH (1980) Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* L. Heynh Theor Appl Genet 58: 257-263

Lange T (1994) Purification and partial amino-acid sequence of gibberellin 20-oxidase from *Cucurbita maxima* endosperm. Planta 195: 108-115

Lange T (1997) Cloning gibberellin dioxygenase genes from pumpkin endosperm by heterologous expression of enzyme activities in *Escherichia coli*. Proc Natl Acad Sci USA 94: 6553-6558

Lange T (1998) Molecular biology of gibberellin synthesis. Planta 204: 409-419

Lange T, Graebe JE (1993) Enzymes of gibberellin synthesis. In: Lea PJ (*ed*) Methods in plant biochemistry, Vol 9. Academic Press, London, pp 403-430

Lange T, Hedden P, Graebe JE (1993b) Gibberellin biosynthesis in cell-free extracts from developing *Cucurbita maxima* embryos and the identification of new endogenous gibberellins. Planta 189: 350-358

Lange T, Hedden P, Graebe JE (1994b) Expression cloning of a gibberellin 20-oxidase, a multifunctional enzyme involved in gibberellin biosynthesis. Proc Natl Acad Sci USA 91: 8552-8556

Lange T, Robatzek S, Frisse A (1997b) Cloning and expression of a gibberellin 2β,3β-hydroxylase cDNA from pumpkin endosperm. Plant Cell 9: 1459-1467

Lange T, Schweimer A, Ward DA, Hedden P, Graebe JE (1994a) Separation and characterization of three 2-oxoglutarate-dependent dioxygenases from *Cucurbita maxima* L. endosperm involved in gibberellin biosynthesis. Planta 195: 98-107

Lester DR, Ross JJ, Davies PJ, Reid JB (1997) Mendel's stem length gene (le) encodes a gibberellin 3β-hydroxylase. Plant Cell 9: 1435-1443

Lester DR, Ross JJ, Smith JJ, Elliott RC, Reid JB (1999) Gibberellin 2-oxidation and the SLN gene of *Pisum sativum*. Plant J 19: 65-73

Lichtenthaler HK, Rohmer M, Schwender J (1997) Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants. Physiol Plant 101: 643-652

MacMillan J (1997) Biosynthesis of the gibberellin plant hormones. Nat Prod Rep 14: 221-243

MacMillan J, Suter PJ (1958) The occurrence of gibberellin A_1 in higher plants: Isolation from the seed of runner bean (*Phaseolus multiflorus*). Naturwissenschaften 45:46

MacMillan J, Seaton JC, Suter PJ (1959) A new plant-growth promoting acid-gibberellin A_5 from the seed of *Phaseolus multiflorus*. Proc Chem Soc 325

MacMillan J, Seaton JC, Suter PJ (1960) Isolation of gibberellin A_1 and gibberellin A_5 from *Phaseolus multiflorus*. Tetrahedron 11: 60-66

MacMillan J, Seaton JC, Suter PJ (1962) Isolation and structures of gibberellin A_6 and gibberellin A_8. Tetrahedron 18: 349-355

MacMillan J, Takahashi N (1968) Proposed procedure for the allocation of trivial names to the gibberellins. Nature 217: 170-171

MacMillan J, Ward DA, Phillips AL, Sánchez-Beltrán MJ, Gaskin P, Lange T, Hedden P (1997) Gibberellin biosynthesis from gibberellin A_{12}-aldehyde in endosperm and embryos of *Marah macrocarpus*. Plant Physiol 113: 1369-1377

Martin DN, Proebsting WM, Hedden P (1997) Mendel's dwarfing gene: cDNAs from the *le* alleles and function of the expressed proteins. Proc Natl Acad Sci USA 94: 8907-8911

Martin DN, Proebsting WN, Hedden P (1999) the SLENDER gene of pea encodes a gibberellin 2-oxidase. Plant Physiol 121: 775-781

Martin DN, Proebsting WM, Parks TD, Dopugherty WG, Lange T, Lewis MJ, Gaskin P, Hedden P (1996) Feed-back regulation of gibberellin biosynthesis and gene expression in *Pisum sativum* L. Planta 200: 159-166

Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, et al. (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous plants. Plant Cell Physiol 37: 49-59

Monna L, Kitazawa N, Yoshino R, Susuki J, Masuda H, Maehara Y, Tanji M, Sato, M, Nazu S, Minobe Y (2002) Postional cloning of rice semidwarf gene, sd1: rice"green revolution gene" encodes a mutant enzyme involved in gibberellin synthesis. DNA Res 9: 11-17

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 473-497

Niki T, Nishijima T, Nakayama M, Hisamatsu T, Oyama-Okubo N, Yamazaki H, Hedden P, Lange T, Mander LN, Koshioka M (2001) Production of dwarf lettuce by overexpressing a pumpkin gibberellin 20-oxidase gene. Plant Physiol 126: 965-972

Oda A, SAkuta C, Masuda S, Mizoguchi T, Kamada H, Satoh S (2003) Possible involvement of leaf gibberellins in the clok-controlled expression of *XSP30*, a gene encoding a xylem sap lectin, in cucumber roots. Plant Physiol 133: 1779-1790

Olszewski N, Sun T-p, Gubler F (2002) Gibberellin signalling: Biosynthesis, catabolism, and response pathways. Plant Cell 14: S61-S80

Peng J, Richards DE, Hartley NM, Murphy GP, Devos KM, Flintham JE, Beales J, Fish LJ, Worland AJ, Pelica F, et al. (1999) Green revolution“ genes encode mutant gibberellin response modulators. Nature 400: 256-261

Phillips AL (2004) Genetic and transgenic approaches to improving crop performance. In Plant Hormones: Biosynthesis, Signal Transduction, Action! (Davies PJ, ed) Dordrecht: Kluwer Academic, pp. 582-609

Phillips AL, Ward DA, Uknes S, Appleford NEJ, Lange T, Huttley AK, Gaskin P, Groebe JE, Hedden P (1995) Isolation and expression of three gibberellin 20-oxidases cDNA clones from *Arabidopsis*. Plant Physiol 108: 1049-1057

Phinney BO, West CA, Ritzel MB, Neely PM (1957) Evidence for gibberellin-like substances from flowering plants. Proc Natl Acad Sci USA 43: 398-404

Prediger EA (2001) Quantitating mRNAs with relative and competitive RT-PCR. Methods Mol Biol 160: 49-63

Prescott AG (1993) A dilemma of dioxygenases (or where biochemistry and molecular-biology fail to meet). J Exp Bot 44: 849-861

Prescott AG, John P (1996) Dioxygenases: Molecular structure and role in plant metabolism. Annu Plant Physiol Plant Mol Biol 47: 245-271

Radley M (1956) Occurrences of substances similar to gibberellic acid in higher plants. Nature 178: 1070-1071

Rebers M, Kaneta T, Kawaide H, Yamaguchi S, Yang YY, Imai R, Sekimoto H, Kamiya Y (1999) Regulation of gibberellin biosynthesis genes during flower and early fruit development of tomato. Plant J 17: 241-250

Richards DE, King KE, Ait-Ali T, Harberd NP (2001) How gibberellin regulates plant growth and development: a molecular genetic analysis of gibberellin signalling. Annu Rev Plant Physiol Plant Mol Biol 52: 67-88

Rodrigo MJ, Garcia-Martinez JL, Santes CM, Gaskin P, Hedden P (1997) The role of gibberellins A_1 and A_3 in fruit growth of *Pisum sativum* L. and the identification of gibberellins A_4 and A_7 in young seeds. Planta 201: 446-455

Ross JJ, Reid JB, Swain SM, Hasan O, Poole AT, Hedden P, Willis CL (1995) Genetic regulation of gibberellin deactivation in Pisum. Plant J 7: 513-523

Saito T, Abe H, Yamane H, Sakurai A, Murofushi N, Takio K, Takahashi N, Kamiya Y (1995) Purification and properties of ent-kaurene synthase B from immature seeds of pumpkin. Plant Physiol 109: 1239-1245

Sakai M, Sakamoto T, Saito T, Matsuoka M, Tanaka H, Kobayashi (2003) Expression of novel rice gibberellin 2-oxidase gene is under homeostatic regulation by biologically active gibberellins. Plant J 116: 161-164

Sakamoto T, Kamiya N, Ueguchi-Tanaka M, Iwahori S, Matsuoka M (2001) KNOX homeodomain protein directly suppresses the expression of a gibberellin biosynthetic gene in the tobacco shoot apical meristem. Genes Dev 15: 581-590

Sakamoto T, Kobayashi M, Ihoh H, Tagiri A, Kayano T, Tanaka H, Iwahori S, Matsuoka M (2001) Expression of a gibberellin 2-oxidase gene around the shoot apex is related to phase transition in rice. Plant Physiol 125: 1508-1516

Sakamoto T, Marinaka Y, Ishiyama K, Kobayashi M, Ihoh H, Kayano T, Iwahori S, Matsuoka M, Tanaka H (2003) Genetic manipulation of gibberellin metabolism in transgenic rice. Nat Biotechnol 21: 909-913

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York

Sazaki A, Ashikari M, Uegushi-Tanaka M, Kobayashi M, Itoh H, Nishimura A, Swapan D, Ishiyama K, Saito T, Klush GS, Kitano H, Matsuoka M (2002) A mutant gibberellin-synthesis gene in rice. Nature 416: 701-702

Schomburg FM, Bizzell CM, Lee DJ, Zeevaart JAD, Amasino RM (2003) Overexpression of a novel class of gibberellin 2-oxidases decreases gibberellin levels and creates dwarf plants. Plant Cell 15: 151-163

Shen WJ, Forde BG (1989) Efficient transformation of *Agrobacterium* spp by high-voltage electroporation. Nucl Acids Res 17: 8385-8385

Smith MW, Yamaguchi S, Tahar AA, Kamiya Y (1998) The first step of gibberellin biosynthesis in pumpkin is catalysed by at least two copalyl diphosphate synthases encoded by differentially regulated genes. Plant Physiol 118: 1411-1419

Spielmeyer W, Ellis MH, Chandler P (2002) Semidwarf (*sd-1*), "green revolution" rice, contains a defective gibberellin 20-oxidase gene. Pro Natl Acad Sci USA 99: 9043-9048

Sponsel VM, Hedden P (2004) Gibberellin biosynthesis and inactivation. In Plant Hormones: Biosynthesis, Signal Transduction, Action! (Davies PJ, ed). Dordrecht: Kluwer Academic, pp. 63-94

Spray CR, Kobayashi M, Suzuki Y, Phinney BO, Gaskin P, MacMillan J (1996) The dwarf-1 (*d1*) mutation of *Zea mays* blocks three steps in the gibberellin biosynthetic pathway. Proc Natl Acad Sci USA 93: 10515-10518

Sun T-p, Kamiya Y (1994) The Arabidopsis GA1 locus encodes the cyclase ent-kaurene synthetase A of gibberellin biosynthesis. Plant Cell 6: 1509-1518

Sun T-p, Goodman HM, Ausubel FM (1992) Cloning the *Arabidopsis GA1* locus by genomic subtraction. Plant Cell 4: 119-128

Takahashi M, Kamiya Y, Takahashi N, Graebe JE (1986) Metabolism of gibberellins in a cell-free system from immature seeds of *Phaseolus vulgaris* L. Planta 168: 190-199

Takahashi N, Kitamura H, Kawarada A, Seta Y, Takai M, Tamura S, Sumiki Y (1955) Biochemical studies on "bakanae" fungus. Part XXXIV. Isolation of gibberellins and their properties. Bull Agric Chem Soc Jpn 19: 267-277

Takahashi N, Seta Y, Kitamura H, Sumiki Y (1957) A new gibberellin, gibberellin A4. Bull Agric Chem Soc Japan 21: 396-398

Talón M, Koornneef M, Zeevaart JAD (1990) Endogenous gibberellins in *Arabidopsis thaliana* and possible steps blocked in the biosynthetic pathways of the semi-dwarf *ga4* and *ga5* mutants. Proc Nalt Acad Sci USA 87: 7983-7987

Tanimoto E (1994) Interaction of gibberellin A_3 and ancymidol in the growth and cell wall extensibility of dwarf pea roots. Plant Cell Physiol 35: 1019-1028

Thomas SG, Phillips AL, Hedden P (1999) Molecular cloning and functional expression of gibberellin 2-oxidases, multifunctional enzymes involved in gibberellin deactivation. Proc Natl Acad Sci USA 96: 4698-4703

Toyomasu T, Kawaide H, Mitsuhashi W, Inoue Y, Kamiya Y (1998) Phytochrome regulates gibberellin biosynthesis during germination of photoblastic lettuce seeds. Plant Physiol 118: 1517-1523

Ueguchi-Tanaka M, Itoh H, Oyama N, Koshioka M, Matsuoka M (1998) Over-expression of a tobacco homeobox gene *NTH15* decreases the expression of gibberellin biosynthetic gene encoding GA 20-oxidase. Plant J 15: 391-400

Vidal AM, Gisbert C, Talon M, Primo-Millo E, Lopez-Diaz I, Garcia-Martinez JL (2001) The ectopic overexpression of a citrus gibberellin 20-oxidase enhances the non-13-hydroxylation pathway of gibberellin biosynthesis and induces an extremely elongated phenotype in tobacco. Physiol Plant 112: 251-260

White OE (1917) Studies of inheritance in Pisum. II. The present state of knowledge of heredity and variation in peas. Proc Am Philos Soc 56: 487-588

Wilkie S (1996) Isolation of total genomic DNA. In: clark MS, plant molecular biology-a laboratory manual. Springer Heidelberg, pp 8-9

Williams J, Phillips AL, Gaskin P, Hedden P (1998) Function and substrate specificity of the gibberellin 3β-hydroxylase encoded by the *Arabidopsis GA4* gene. Plant Physiol 117: 559-563

Wong H, Anderson WD, Cheng T, Riabowol T (1994) Monitoring mRNA expression by polymerise chain reaction: the “primer dropping” method. Anal Biochem 223: 251-258

Xu YL, Gage DA, Zeevaart JAD (1997) Gibberellins and stem growth in *Arabidopsis thaliana* – Effects of photoperiod on expression of the GA_4 and GA_5 loci. Plant Physiol 114: 1471-1476

Xu YL, Li L, Gage DA, Zeevaart JAD (1999) Feedback regulation of GA_5 expression and metabolic engineering of gibberellin levels in *Arabidopsis*. Plant Cell 11: 927-935

Xu YL, Li L, Wu K, Peeters AJM, Gage DA, Zeevaart JAD (1995) The *GA5* locus of *Arabidopsis thaliana* encodes a multifunctional gibberellin 20-oxidase: molecular cloning and functional expression. Proc Natl Acad Sci USA 92: 6640-6644

Yabuta T (1935) Biochemistry of the “bakanae” fungus of rice. Agriculture and Horticulture 10: 17-22

Yabuta T, Sumiki Y (1938) On the crystal of gibberellin, a substance to promote plant growth. J Agric Chem Soc Japan 14: 1526

Yamaguchi S, Kamiya Y (2000) Gibberellin biosynthesis: its regulation by endogenous and environmental signals. Plant Cell Physiol 41: 251-257

Yamaguchi S, Kamiya Y, Sun T-p (2001) Distinct cell-specific expression patterns of early and late gibberellin biosynthetic genes during *Arabidopsis* seed germination. Plant J 28: 443-453

Yamaguchi S, Saito T, Abe H, Yamane H, Murofushi N, Kamiya Y (1996) Molecular cloning and charaterization of a cDNA encoding the gibberellin biosynthetic enzyme ent-kaurene synthase B from pumpkin (*Cucurbita maxima* L.). Plant J 10: 203-213

Yamaguchi S, Smith MW, Brown RGS, Kamiya Y, Sun T-p (1998) Phytochrome regulation and differential expression of gibberellin 3β-hydroxylase genes in germinating *Arabidopsis* seeds. Plant Cell 10: 2115-2126

Yaxley JR, Ross JJ, Sherriff LJ, Reid J (2001) Gibberellin biosynthesis, mutation and root development in pea. Plant Physiol 125: 627-633

7. Appendix

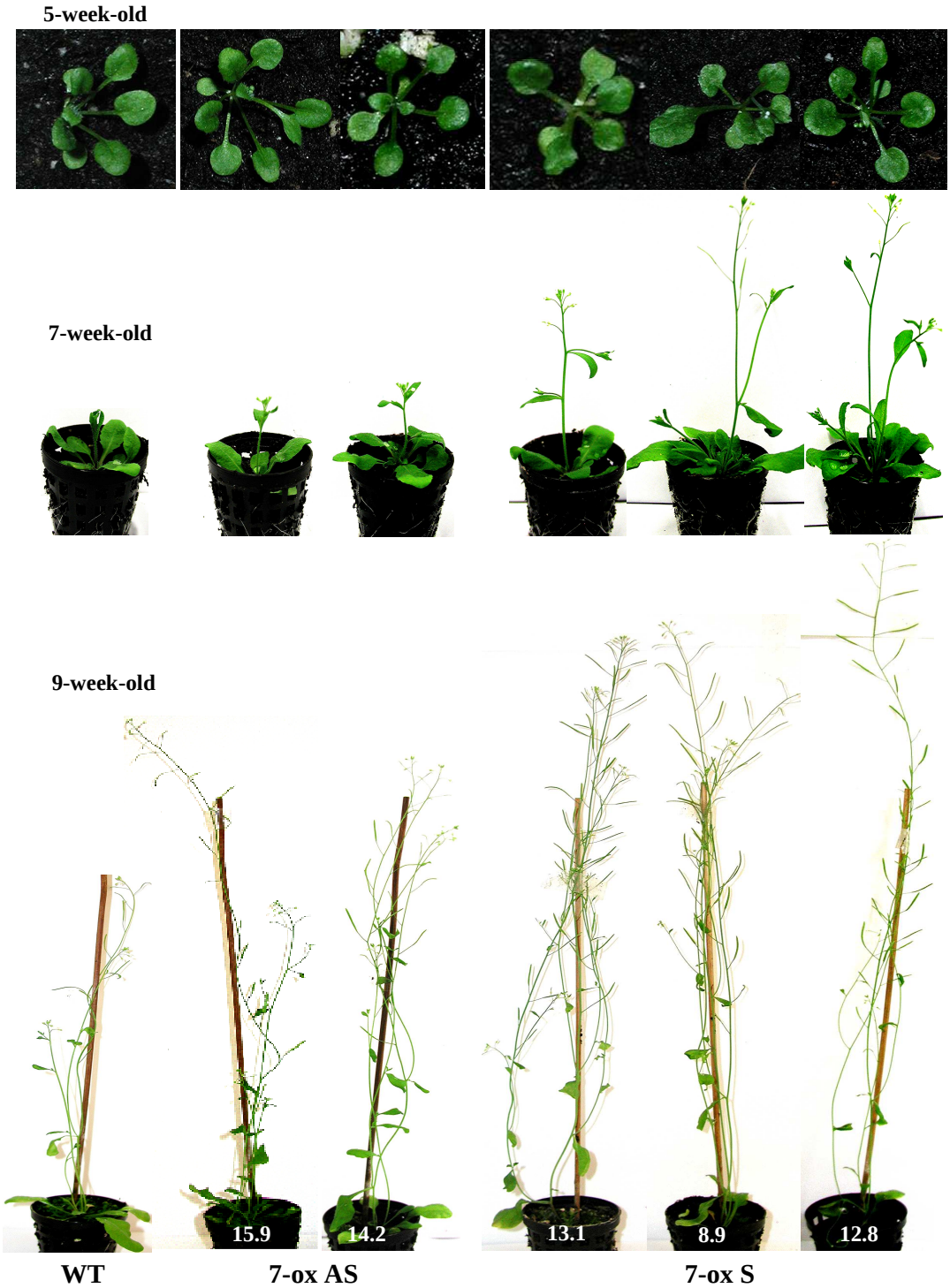


Figure 7.1: Plant development of sense (S) or antisense (AS) copies of pumpkin 7-ox lines in *Arabidopsis* plants. Wild type plants (WT) are displayed as control.

Figure 7.2: Plant development of sense (S) copies of pumpkin 20-ox-1 lines in *Arabidopsis* plants. Wild type plants (WT) of the respective developmental stage are displayed as control.

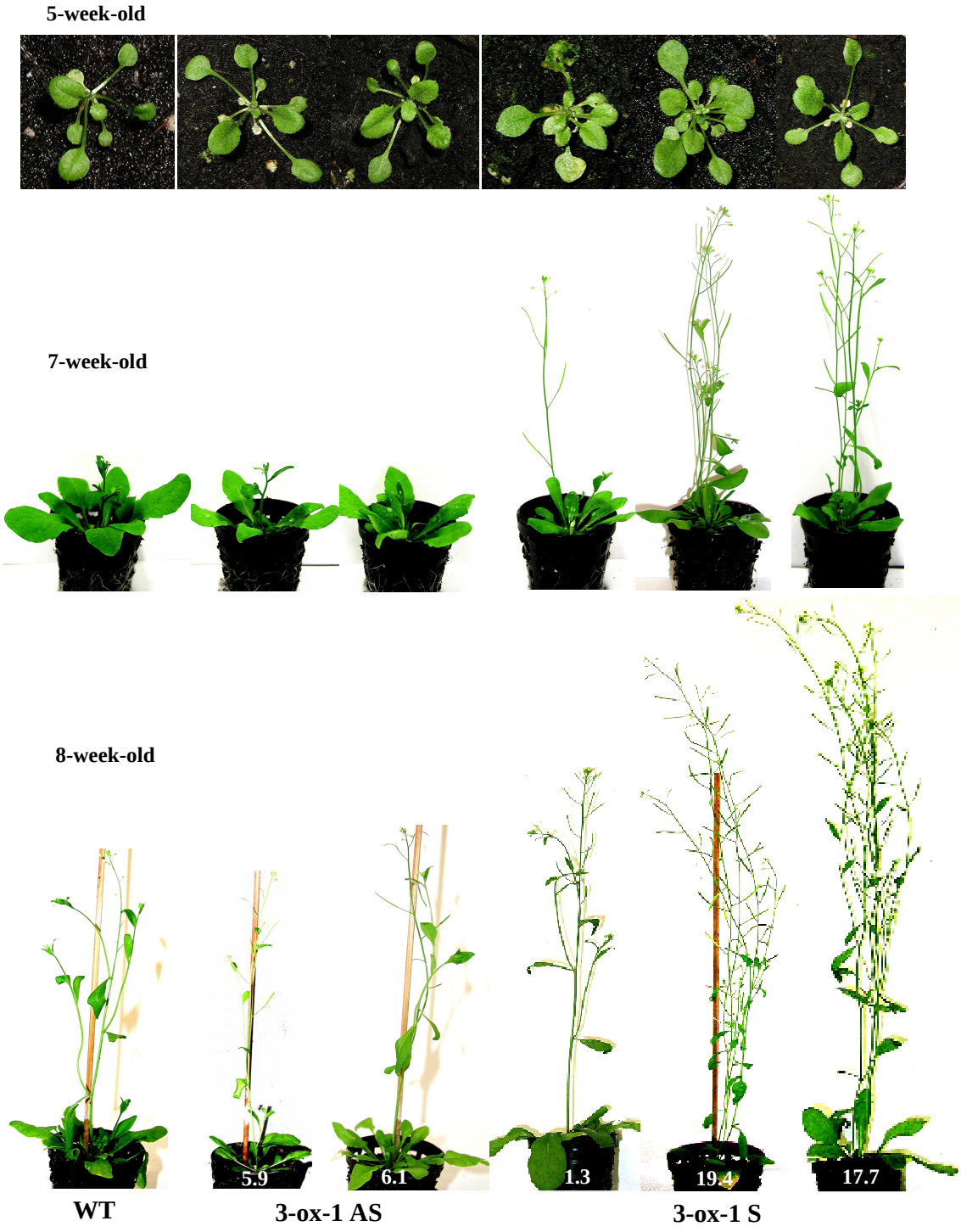


Figure 7.3: Plant development of sense (S) or antisense (AS) copies of pumpkin 3-ox-1 lines in *Arabidopsis* plants. Wild type plants (WT) of the respective developmental stage are displayed as control.

Figure 7.4: Plant development of sense (S) or antisense (AS) copies of pumpkin 2-ox lines in *Arabidopsis* plants. Wild type plants (WT) of the respective developmental stage are displayed as control.

Curriculum Vitae
of Abeer A.F.M. Radi

Name : Abeer Ahmad Farag Mahmoud Radi

Date of birth : January 8, 1969

Nationality : Egyption

Marital status : Married, having two sons: Omar born on 12/08/1992 and Ali born on 22/03/1997

Present address : Deparment of Botany, Faculty of Science, Assiut University, Assiut, Egypt

Telephone, Residence : 002-088-326728 or 002-088-334270

Fax : 002-088-327254 or 002-088-312564

Education

1974-1980	Primary school, Assiut, Egypt
1980-1983	Preparatory school, Assiut, Egypt
1983-1986	Secondary School, Assiut, Egypt
1986-1990	Faculty of Science, Assiut, Egypt

Academic qualifications:

- B.Sc. Botany, Assiut University, June 1990 “Distinction with honour”

- M.Sc. Botany (plant physiology), Assiut University, September 1996
  Title of M.Sc. thesis: “Counteraction of salinity stress on some plants by seed presoaking in nicotinic acid or gibberellic acid”

Employment record:

- Demonstrator, of plant physiology, Department of Botany, Faculty of Science, Assiut University, July 1990

- Assistant lecturer, of plant physiology, Department of Botany, Faculty of Science, Assiut University, October 1996